The *Pax6* gene in the development of the eyes, nose and brain.

Justin C. Grindley

Ph. D

University of Edinburgh

1995

Acknowledgements

I have never managed to be much good at doing things that I did not choose to do. For me, Bob Hill's strength as a supervisor has been in giving me freedom to study what interested me and to choose how to study it, but he has also managed to be involved and helpful when needed. Bob has put his own work aside to sort out problems with my project, and he rescued me from a great deal of frustration by performing the PCR genotyping of my embryos. Thanks Bob.

There is also a lot to I want to thank Duncan Davidson for, but I mainly want to highlight Duncan's willingness to pass on his considerable expertise. Most of my grasp of embryology comes from Duncan, but the errors are, of course, my own. Virtually all the techniques I have used have been learnt from Duncan or from someone else in the lab. I will not have space to mention everyone, but I have not forgotten their help. Liz Graham taught me how to do *in situ* hybridisations, and ran a tight *in situ* ship, which made the experiments a lot more successful. I did my first wholemount *in situ* hybridisations with Lesley McInnes, and thanks to her they worked straight away, something which would never, ever have happened if I had done them myself. My struggles with sections were sorted out by Allyson Ross, with lots of patient advice on all things histological. This was supplemented by tips and methods from Corrine Arnott and from everyone in Wolfgang Schmahl's group. Andrew Ross took my samples and prepared them for electron microscopy, taught me how to use the electron microscopes and developed the results, so most of the credit for this work should go to him. Anne generally ran the lab, and Lorna made a little bit of sense out of the infamous *in situ* probe-box. The whole project would have gone nowhere without Donald Hay and everyone at the Biomedical Research Facility, but I especially want to thank Vince for all his work over the years.

The computer presentation of the *in situ* results was made possible by Richard Baldock. He and John Ireland also sorted out many computer glitches. My photography was made presentable by the work of Norman Davidson, Sandy Bruce and Douglas Stuart. In the library, Sheila Mould tracked down many references which made a huge difference to my work and my thesis. All those sterile solutions

were there when I needed them thanks to everyone in wash-up, who never seem to get half the credit they deserve.

Lots more people in the MRC have helped out in one way or another and made the whole Ph.D experience more enjoyable. David, Anne, Richard, Maureen, Paula, Paula and especially Lesley made me feel welcome when I first arrived. Sarah, Kathy Williamson and Adrian shared an office with me, watered my poor plants when I was away, and didn't complain too much about the mess. David Townley and David Hughes played the right music (usually). Mention must also be made of those I faced the horrors of the Western General Hospital's crap food with, especially Sinéad. I haven't been there for ages, it's just too awful without some mutual support.

Lots of people really have helped by taking the time to talk, listen and offer advice. Jonathan persuaded me to stick with this Ph.D after a wasted first year. The support, humour and interest of other students has been important, so to those mentioned already, let me add Vasker, Jill, Martin and Jem. Ian and Colin both have an infectious enthusiasm for science, which has helped along the way, and it was good to have someone around as lively and enthusiastic about eye development as Sophie. Veronica would not just talk about experiments, but go and get the antibodies and hassle me into doing them. She also gets a special mention for her concern for my welfare. During late nights in the lab there were lifesaving breaks for tea and a chat with those guarding the building, but especially Archie, Adam, Tom and Alastair. I have particularly enjoyed hill walking and playing hockey with people from the lab, and joining them in the main MRC social activity, going to the pub. Paul and Vasker have always been ready to go to the pub, and Stewart really has bought me lots of pints, despite appearances.

Bridgeen got me into this mess in the first place. Dave, Paul, Martin and Gaynore have been good friends throughout and provided places to stay when I needed to get away from it all. The friendship of my flatmates in Edinburgh over the years has meant a lot, and they have put up with me and my Ph.D more than most. I'd like to mention Vasker for getting to the tops of many mountains with me, Isabelle for sharing her philosophies on life and love, and Ruth for understanding and

boundless energy. David gets a mention here too, for offering somewhere to stay when I need it most. Thanks also to Leith DSS.

In my international acknowledgements section I ought to thank those who sent me probes, but instead I want to mention Jack Favor's hospitality during in my time in Neuherberg, Wolfgang Schmahl for making brain development seem so fascinating, and Brigid whose job offer was a prime motivation to get the writing finished.

The last and most important thanks of all go to my family, who supported me in every way possible.

Previously published material.

Part of the work presented in this thesis has been previously published as Grindley *et al.*, (1995). A reprint of this manuscript is included as an appendix.

Abstract.

Small eye (*Sey*) mouse embryos, homozygous for mutations in the *Pax6* gene, have no eyes, no nasal cavities and no olfactory bulbs (Hogan *et al*, 1986; Hill *et al,* 1991). Investigating the basis of this striking phenotype could potentially provide valuable insights into the normal processes involved in the development of the eye, nose and brain. *Pax6* encodes a transcription factor, with two DNA-binding motifs, a paired domain and a *paired*-type homeodomain (Walther and Gruss, 1991). The work described in this thesis is an investigation into the potential roles of this regulatory gene during normal development. Information obtained from studying the pattern of *Pax6* mRNA expression in wild-type embryos, and the developmental defects of *Sey/Sey* animals, was combined to identify processes that may involve PAX6.

PAX6 is essential for the formation of lens placodes from surface ectoderm. In normal development, early *Pax-6* mRNA expression in a broad domain of surface ectoderm is downregulated, but expression is specifically maintained in the developing lens placode. Thus, phenotype and expression together suggest a role for PAX6 in lens determination. In surface ectoderm in the eye region, PAX6 function is also required for the maintenance of *Pax6* transcription.

Like the lens, the nasal cavities develop from ectodermal placodes that normally express *Pax6* mRNA, fail to form in *Sey/Sey* mice and show PAX6 dependent *Pax6* mRNA regulation. Analysis of patterns of programmed cell death and absence of nasal region expression from an *Msx1* transgene in *Sey/Sey* embryos, suggest a requirement for Pax-6 in the transition from presumptive nasal ectoderm to placode, and that *Msx1,* or genes regulating it, are possible targets for PAX6.

Sey/Sey animals have defects early in forebrain development, manifested by the swollen appearance of the prosencephalon and a failure of the proximal optic vesicles to constrict. These phenotypes may derive in part from a failure of nasal development, or from hydrocephaly, but their relationship to *Pax6* mRNA expression patterns suggest some localised requirement for PAX6 function within the developing forebrain.

Structures particularly affected in *Sey/Sey* embryos, such as the telo-diencephalic sulci and the constricting proximal optic vesicle, are sites of strong *Pax6* expression. This correlation, and the variation of *Pax6* expression levels within the folding neuroepithelium, suggests that PAX6 may regulate some cell behaviour necessary for normal folding.

Pax6 mRNA expression in the diencephalon respects neuromere boundaries, raising the possibility that altered brain shape in mutants results from abnormal antero-posterior specification. Presence of normal morphological boundaries argues against a role for PAX6 in defining segments in the posterior diencephalon, but this remains a possibility for more anterior segments.

CONTENTS

CHAPTER 1.

Introduction and aims of the work presented. [pp 1-95]

CHAPTER 3

Results from studies addressing the roles of the *Pax6* gene in eye and nasal development. [pp 141-178]

CHAPTER 6 (continued)

CHAPTER 7
Suggested improvements and possible future studies [pp 241-246]

BIBLIOGRAPHY [pp 247- 277]

APPENDIX

LIST OF FIGURES

CHAPTER 1

CHAPTER 3

CHAPTER 3 (continued)

CHAPTER 5

LIST OF TABLES

CHAPTER 2

CHAPTER 1

Introduction and aims of the work presented.

Overview of the introduction:

Homozygous embryos of the mouse *Small eye* (*Sey*) mutant have no eyes, no nasal cavities and no olfactory bulbs (Hogan *et al.,* 1986). Investigating the basis of this striking phenotype could potentially provide valuable insights into the normal processes involved in the development of the eye, nose and brain. *Small eye* has been shown (Hill *et al.,* 1991) to result from mutations in *Pax6*, a gene encoding a transcription factor, with two DNA-binding motifs, a paired domain and a *paired*-type homeodomain. The work described in this thesis is an investigation into the potential roles of *Pax6* in normal development. The study concentrates on three developmental systems, eye development, nasal development and early forebrain development, in which there is *Pax6* expression (Walther and Gruss,1991) and developmental defects in *Sey/Sey* embryos with *Pax6* mutations (Hogan *et al.,* 1986).

This introduction will first outline the normal structure and development of the tissues that will feature in the expression and phenotypic-analysis experiments. Once normal development has been described, some of the questions raised will be reviewed. The use of molecular biology techniques and of genetic approaches are powerful tools in the analysis of such problems. The approaches adopted in this study are principally the analysis of gene expression patterns and mutant phenotypes. The types of information that may be obtained in this way is considered along with how genes involved in processes of interest might be obtained.

The Pax genes (Walther *et al.,* 1991) encode a class of developmentally important transcription factors. Some general features of the family are followed by a series of resumes for each of the mammalian Pax genes for which roles have been

suggested. More detail is included for the last of these to be considered, *Pax6* itself. Molecular studies of special relevance to the question of PAX6 function are by no means confined to the analysis of *Small eye* mice. In humans, *PAX6* has been shown to be deleted or mutated in defects of the anterior segment of the eye, human aniridia (Ton *et al.,* 1991; Glaser *et al.,* 1992; Hanson *et al.,* 1993; Jordan *et al.,* 1992), and Peters anomaly, (Hanson *et al.,* 1994a) characterised by central corneal opacity. Rat *Small eye* also results from *Pax6* mutations with similar phenotypic consequences to the mouse mutant (Matsuo *et al.,* 1993). The nature of the human, mouse and rat mutations and their mode of inheritance will be considered together.

General properties, common to a number of Pax genes are identified, with an emphasis on the features of the roles they may have during development, including the targets of Pax genes and how Pax genes are regulated.

When the work described in this thesis was initiated, little was known about PAX6 function or its expression during development. The penultimate parts of the introduction will review what was known about *Pax6* at the time and the questions the study aimed to address. The final section of introduction describes the experimental approach adopted to address these questions, which was a combined analysis of the mRNA expression of *Pax6* during development and the developmental defects of *Sey/Sey* embryos with *Pax6* mutations.

Normal structure and development of the visual system, olfactory system and early forebrain.

Descriptions of normal development

The descriptions in these sections will necessarily be highly selective, considering in particular those processes that *Pax6* mutations may disrupt, especially around the time of the first appearance of *Pax6* expression and *Sey/Sey* defects. Some of the interactions between tissues in each system will also be considered. If secondary defects result, such interactions between tissues can complicate phenotypic analyses, but defects in interacting tissues may also be revealing about the nature of the interactions. The experimental analysis of disrupted development in *Sey/Sey* embryos has been entirely prenatal, since *Sey/Sey* mice die at birth (Hogan *et al.*, 1986). Late gestational and postnatal development of some structures will however be briefly considered. Heterozygosity for *Pax6* mutations is associated with developmental defects in some late developing structures such as the iris (Ton *et al.*, 1991; Glaser *et al.*, 1992; Jordan *et al.*, 1992; Hanson *et al.*, 1993) and cerebral cortex (Schmahl *et al.*, 1993), which may also highlight important roles for *Pax6* in development.

1.1 Structure and development of the visual system.

1.1.1 The structure of the mammalian eye.

There are a variety of different evolutionary solutions to the problem of extracting visual information from the environment (reviewed by Land, 1992). In vertebrates this is done by producing a focused image on a photosensitive retina which produces neuronal signals in response to light (general reference Gray's Anatomy, 1973; Kahle, 1986). The apparent simplicity of this description conceals the highly specialised nature of this task and the many unique features consequently required of ocular tissues. A schematic structure of an adult mammalian eye, is shown in Figure 1.1.

Focusing of the image begins as light enters the eye through the curved transparent cornea, with much of the necessary refraction of the light occurring at the air-cornea boundary. Behind the cornea is the anterior chamber, which like the posterior chamber behind the iris, is filled with aqueous humor. The aqueous humor maintains intraocular pressure and is also mediates the supply of glucose and amino acids and the exchange of respiratory gases for the avascular tissues, the lens and central cornea

In front of the lens is the iris, a diaphragm arrangement controlling the amount of light entering the interior of the eye. This is done by the action of pupillary sphincter and pupillary dilator muscles in the iris altering the size of its central aperture, the pupil.

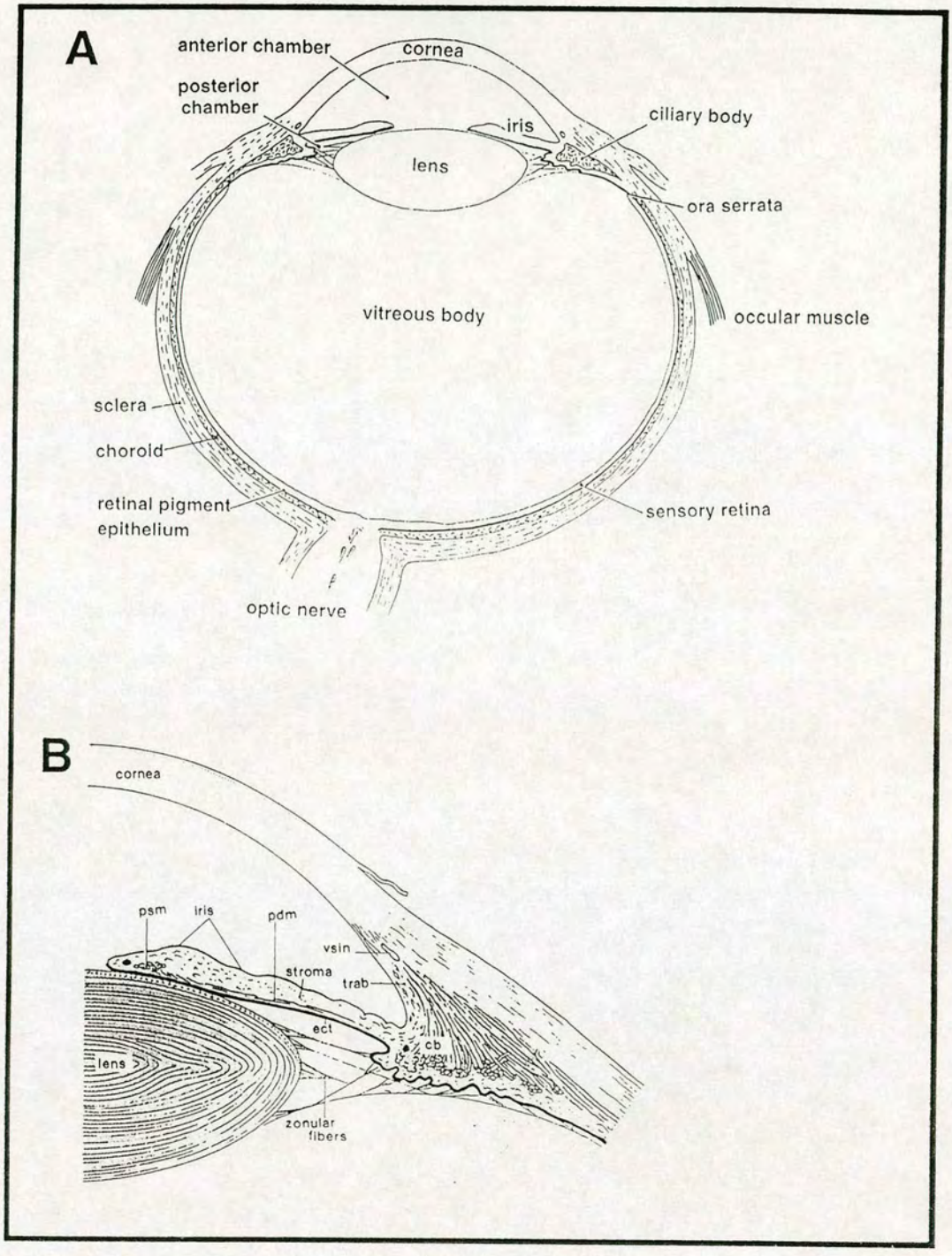

Figure 1.1

Structure of the mammalian eye. A) Cross section of the adult eyeball showing principal structures and chambers. B) Detail of angle of the eye and anterior chamber. cb, ciliary body; trab, trabecular meshwork; psm, pupillary sphincter muscle; pdm, pupillary dilator muscle; ect, ectodermal layer of iris; vsin, venous sinus. Based on illustrations in Kahle (1986).

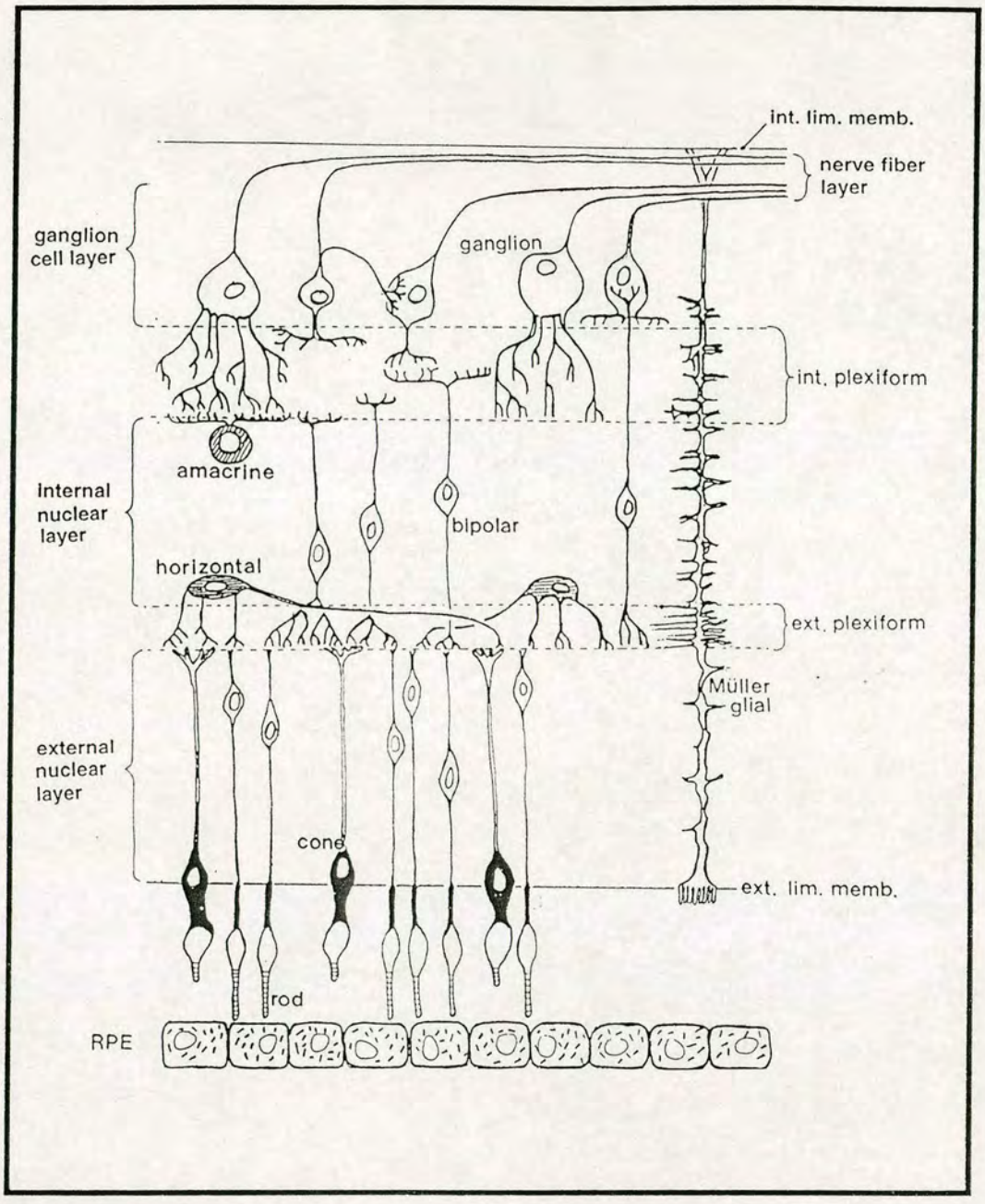

Figure 1.2

Laminar structure of the retina, with main cell types and typical connections. The outer segments of rod and cone photoreceptors project through the external limiting membrane (ext. lim. membrane), coming in close proximity to the cells of the retinal pigment epithelium (RPE). Photoreceptors make connections with the horizontal and bipolar cells within the external plexiform layer (ext. plexiform). Nuclei of horizontal, bipolar and amacrine cells make up the internal nuclear layer. Amacrine and bipolar cells contact the dendrites of ganglion cells within the internal plexiform layer (int. plexiform). Ganglion cell axons running to exit the eye via the optic nerve form the nerve fibre layer. Müller glia cells stretch between the outer limiting membrane and internal limiting membrane (int. lim. memb.), and have multiple fine processes branching laterally.(Based on an illustration in Kahle ,1986)

Further focusing and variation of the plane of focus, is performed by the lens, a biconvex structure of elongated epithelial cells. Variation of plane of focus, termed accommodation, is possible as the lens is not rigid, but rather can be flattened by the action of muscles of the ciliary body, to which the perimeter of the lens is connected by a suspensory cradle of thin fibres.

The interior chamber of the eye, almost spherical in mammals, is filled with a watery gel, the vitreous body, bound by the hyaloid membrane. Light leaving the lens passes through the vitreous body and falls on the retina which lines the interior chamber as far anterior as the ora serata. The retina has at least eight distinct cell types; retinal pigment epithelium cells, two types of photoreceptors, Muller(glial)cells, and four types of neuronal cells, the bipolar cells, horizontal cells, ganglion cells and the axon-less amacrine cells. These are arranged in a layered structure in which cell bodies form nuclear layers, separated by plexiform layers with many cell processes but few nuclei. A simplified diagram of the retinal structure is shown in Figure 1.2.

The outermost layer of the retina is the retinal pigment epithelium (RPE), a layer of closely packed cuboidal epithelial cells containing melanosomes, vesicle bound organelles containing melanin pigment. This pigment serves to reduce reflections within the eye. The RPE probably also has a transport function in supplying nutrients to the retina from the vascular choroid underlying the RPE. The outer segments of the photoreceptor cells project from the external limiting lamina and come into close association with RPE cells. This arrangement of photoreceptors

means that light must pass through all the inner layers of retinal cells before triggering the photoreceptors.

There are many times more photoreceptors than neurons in the retina, hence large numbers of them must activate a single axonal pathway. Horizontal cells connect either rods or cones over long ranges, up to 1 mm in humans. Bi-polar cells are the primary sensory neurons on the retino-tectal pathway, with dendrites to rods and cones and axons that synapse with ganglion cells. Amacrine neurons interconnect with dendrites of ganglion cells and axons of bi-polar neurons. Ganglion cells produce non-myelinated axons that enter nerve fiber layer and are directed towards the optic disc to form the optic nerve

To leave the eye the optic nerve must pass through a gap in the choroid and scleral layers. The sclera is a dense collagen-rich connective tissue surrounding the eye. It is smooth to allow movement within the orbits, and yet capable of withstanding the intra ocular pressure and of preventing the eye from distorting as ocular muscles attached to it move the eyeball.

Forming a vertebrate eye requires some cell types to have unusual properties that are not required in any other tissue, such as transparency and a correct refractive index. The relative arrangement of the ocular tissues is also vital for correct function, for example for a focused image to the formed the lens must be oriented in the correct plane and accurately placed relative to the retina, at a distance appropriate for its focal length. These precise arrangements are achieved in part by regulatory interactions between the tissues forming the eye.

1.1.2 Normal development of the eye

1.1.2.1 Contributions from ectoderm, neural crest and mesoderm.

The normal development of the eye has been reviewed by a number of authors (Coulombre, 1965; Kaufman, 1979, 1992; Barishak, 1992). The eye is formed from ectodermal and mesenchymal cell types acting in concert. The early eye is formed from two interacting tissues, the neural ectoderm which forms the retina and optic stalk, and the surface ectoderm which forms the lens and corneal epithelium. Slightly later, a contribution is apparent from mesenchymal tissues of either mesodermal or neural crest origin. Interspecific transplant experiments have been used to distinguish between these two possible sources (Johnston *et al.*, 1979). Such studies revealed that neural crest forms scleral and choroidal cells, much of orbit and at, the anterior of the eye, endothelial and stromal cells of cornea, and ciliary muscles. Mesodermal contributions are to the extrinsic ocular motor muscles, periocular vascular endothelial cells.

1.1.2.2 Early events in optic vesicle and lens development

The embryology of the early eye is illustrated schematically in Figure 1.3 with time-scales for the mouse (Rugh, 1968; Pei and Rhodin, 1970; Kaufman, 1979; Theiler, 1989; Kaufman, 1992).

In the mouse the eye can be first detected morphologically at about the four somite stage (corresponding to 8-8.25 days post coitum (E8-8.25)) as a flattened , rather diffuse thickening of the neural ectoderm in the presumptive prosencephalon

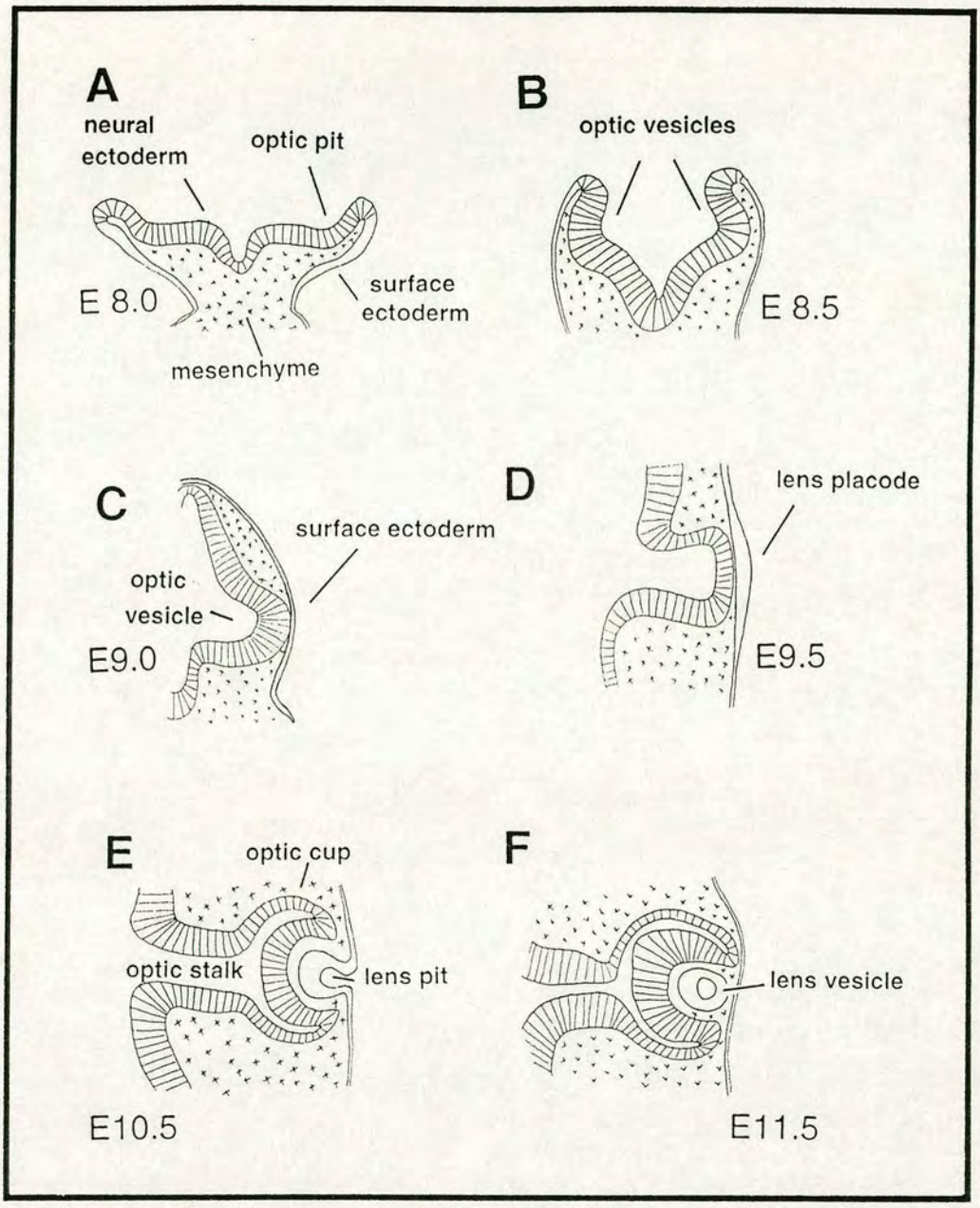

Figure 1.3.
Stages of early eye development in vertebrates, with timings for the mouse.
The early eye is formed from neural ectoderm, which forms the retina and optic stalk, and from surface ectoderm, which forms the lens and cornea. (A). At E8.0 the eyes are detectable as slight indentations of the neural ectoderm, the optic pits. (B) As the head folds close these become the more prominent optic vesicles. (C) Around E9.0 the optic vesicles contact the surface ectoderm. (D) In the area of contact surface ectoderm thickens to form a lens placode. (E) The lens placode invaginated to form a lens pit (by E10.5). The optic vesicle forms an optic cup around the developing lens pit. (F). By E11.5 the lens pit has pinched off to form a lens vesicle, and the two distinct layes of the optic cup are apparent, a thin outer layer, the prospective pigmented retinal epithelium, and the thicker prospective neural retina.

region of cephalic neural plate (Kaufman, 1979). The central region of these thickenings invaginates shortly afterwards (five to six somite stage) forming the optic pits (Fig. 1.3A). As the head fold close, this becomes a more prominent out-pocketing of the brain, the optic vesicle. By E9.0 the lateral optic vesicle has come into contact with surface ectoderm, displacing previously intervening mesenchymal cells (Fig. 1.3 B). The surface ectoderm thickens in the region of contact to form a lens placode from E9.5 to E9.75 (Fig. 1.3 C). Around this time there is a rapid increase in volume of the forebrain, during which the optic vesicles also increase in volume, although there is a reduction in diameter of optic stalks (Kaufman, 1979). The lateral wall of optic vesicle also becomes thicker to form the retinal disc. Studies from other vertebrates suggest that basement membranes of surface ectoderm and optic vesicle become tightly opposed around this stage, with few intervening cells (Johnston *et al.,* 1979). Specifically, there is close contact between thickened basement membranes around the margin of the retinal disc and adjacent lens placode, and a fibrous network linking the center of the retinal disc with the lens placode (Johnston *et al.,* 1979; Yang and Hilfer, 1982).

1.1.2.3 Formation of the lens from lens placode

The lens placode invaginates forming a lens pit (E10.5)(Fig. 1.3 D), which then pinches off to form a lens vesicle (E11-11.5) (Fig 1.3 E), subsequently becoming the lens. As the lens vesicle pinches off, surface ectoderm heals and will form corneal epithelium. A distinct polarity develops in the lens, which may be dependent upon the retina (Yamamoto, 1976). This polarity can already be seen by E10.75 when cells at the back of the lens pit turn on alpha crystallin expression

before those at the rim (Zwaan, 1983). Morphological differences between anterior and posterior lens vesicle cells become apparent at E11.5 to E12 when the cells of the posterior wall become increasingly elongated, with nuclei positioned near their basal surfaces. The pattern of cell division and differentiation apparent at this age is maintained into adulthood, thus at the anterior surface of the lens is a layer of proliferating lens epithelial cells, whereas around the equator of these cells they start to differentiate into lens fiber cells, increasing in volume and elongating (Beebe *et al.,* 1982). This mechanism of lens fiber production means that the oldest lens fibres are found at the center of the lens.

1.1.2.4 Mechanisms of optic cup formation

The face of the optic vesicle adjacent to the lens placode invaginates in parallel with the invagination of the placode. Just how closely tied these two processes are remains unclear. There is certainly some physical connection, as the margin of the optic cup remains attached to the ectoderm at the edge of the lens vesicle (Yang and Hilfer, 1982). Nevertheless, lens vesicle formation can occur in the absence of optic cup formation (Yang and Hilfer, 1982).

The optic vesicle grows around the lens pit and lens vesicle to form a bi-layered optic cup (Fig 1.3D). By E10.5 the two layers can be seen to be distinct thicknesses. The thin outer layer and thicker inner layer of the optic cup will form pigmented retinal epithelium and neural retina respectively.

A variety of mechanisms have been proposed that might contribute to the invagination that forms the optic cup, but not all of these actually do so. Inhibition of glycoconjugate synthesis (Yang and Hilfer, 1982) suggests that one significant factor

is the presence of glycoproteins, normally found on the apical surface of the optic vesicle and in bridges between the two neural ectoderm layers at the anterior boundary of the optic cup. In contrast, whilst patterns of cell death correlate with the invagination process, early optic vesicle formation proceeds normally in *Or* mutant mice that lack this cell death (Silver and Robb, 1979). The invagination could involve changes of cell shape (Svoboa and O'Shea, 1987), driven by cytoplasmic contractile proteins (microfilaments), in an ATP and Ca^{2+} dependent manner (Lee and Auersperg, 1980). Hilfer and co-workers showed that optic vesicle formation is indeed dependent on both ATP and Ca^{2+} uptake (Palmatier and Hilfer, 1977; Brady and Hilfer, 1982)

1.1.2.5 Formation of laminar structure in the neural retina.

The neural ectoderm that forms the neural retina is a pseudostratified single layer of cells with similar proliferative behaviour to the neural tube. Cell bodies undergo interkinetic migration back and forth within the layer, with cell division taking place at the ventricular surface (Sidman, 1961). The interkinetic migration, with division at the former ventricular surface, continues throughout retina development (Young, 1985). Initially both daughters cells from a division re-enter the cell cycle but an increasing proportion migrate away to form the inner layers within which they will differentiate. Thus cells of all later layers are produced in the same ventricular region (Ramon y Cajal, 1960; Sidman, 1961).

The first indications of a laminar structure in the mouse neural retina is the appearance of a narrow, nuclei-free, inner marginal zone at about E12 and the formation of a mantle zone between this and the outer primitive ependymal zone.

Most of the full layer structure develops postnatally, but by about E14 the mantle zone has produced two distinct nuclear (neuroblastic) layers, which are still present at birth (Sidman, 1961). The outer nuclear layer contains ganglion cells and amacrine cells, and the inner nuclear layer contains a mixed population of the future photoreceptors along with Müller, horizontal and bipolar cells of the future bipolar layer. Movement is required to sort out these mixed populations into layers, and cell sorting behavior of different retinal cell-types suggests that homophilic adhesion is involved (Sheffield, 1982).

1.1.2.6 Lineage independence and simultaneous origin retinal cell types.

The ^{3}H-thymidine labeling studies of Sidman (1961) showed that the cell types that will form the photoreceptor, bipolar and ganglion cell layers are all being produced simultaneously, from the same place, but the proportions of different cell types produced varies with time.

Lineage studies (Turner and Cepko, 1987; Holt *et al.,* 1988; Turner *et al.,* 1990) showed that most neural retina progenitor cells were multipotent, which argues against separate precursor populations for different cell types but alternatively favors cell fate being acquired in response to environmental cues. When chick retina cells are isolated early and cultured they form photoreceptors, normally a late developing cell type (Adler and Hatlee, 1989). This suggests that one of the environmental factors present in early retina might be suppressing a 'default' photoreceptor fate to allow other cell types to be formed. It also seems likely that some feedback mechanism exists by which previously formed cells influence the cell type being produced (Reh and Tully, 1986).

1.1.2.7 Non-uniform development of retinal regions and distinct origins of dorsal and ventral retina.

Differentiation of the retina proceeds from the center to the periphery (Coulombre 1955). The peaks of production of the various cell types give an indication of the extent of this effect. Peaks of production of cones and rods in posterior retina occur at E 13-14 and around birth respectively. For both cell types the peaks are two to three days later at the periphery.

There are also a number of differences between the dorsal and ventral retina during development. One visible example is the ventrally forming choroid fissure, the seam along which the nasal and temporal sides of the optic cup fuse. Experiments with eye rotations (Gaze *et al.,* 1979a; Holt, 1980) and cell marking in *Xenopus* (Jacobson and Hiroshi, 1978) suggest that unlike the rest of the retina, cell in the ventral region around the choroid fissure migrate in from the optic stalk. These cells appear to have crossed the midline, possibly suggesting that ventrally the domains destined to form the two eyes were initially contiguous. Since the optic pits appear to be linked across the midline, connection between the ventral presumptive eye forming domains across the midline may also occur in the mouse (Kaufman, 1979).

Differential expression suggests that the some tasks may be performed by different molecules in dorsal and ventral retina. Notably, distinct retinoic acid synthesising activities are present in dorsal and ventral retina (McCaffery *et al.,* 1992). The importance of this difference is shown by selective inhibition of the ventral activity which results in absence of ventral retina (Marsh-Armstrong *et al.,* 1994)

1.1.2.8 Induction of the lens.

For over eighty years the majority view on lens formation focused on the idea that when the optic vesicle came into contact with surface ectoderm, it induced lens formation in the region of contact (for a review of this early work see Spemann, 1938). During this time it was recognised however that lens determination was a multi-step process that might involve several inductive tissues including pharangeal endoderm and heart mesoderm (Jacobson, 1966). Recently there has been a shift in emphasis prompted by a re-evaluation of the experiments which had led to the idea that the optic vesicle was a major lens inducer (Saha *et al.,* 1989; Grainger, 1992). It is now believed that contact with the optic vesicle is not sufficient for lens 'induction' from surface ectoderm, nor is it strictly necessary (reviewed by Jacobson and Sater, 1988; Grainger, 1992), but the optic vesicle does have an important role in supporting the growth and differentiation of the lens (Yamamoto, 1976; Coulombre and Coulombre, 1964).

The optic vesicle was a plausible candidate for a lens inducer. Anatomists had long recognised that lens formation closely followed contact of optic vesicle with surface ectoderm. In experiments performed by Spemann in 1901 (see Spemann, 1938, Jacobson and Sater, 1988), ablation of the prospective optic vesicle without damaging surface ectoderm resulted in a total absence of the eye, with no lens formation. This result suggested that optic vesicle was necessary for lens formation. In bringing optic vesicles together with non-lens ectoderm, lenses were produced, suggesting that contact with an optic vesicle might be sufficient for lens induction (see Spemann, 1938; Saha *et al.,* 1989).

Jacobson (1966), prolonged the contact between the prospective lens epidermis and the pharangeal endoderm of salamanders by explant culture, and found that 31% of explants formed lenses. Along with experimental removal of the endoderm, these experiments suggest that endoderm is an important lens inducer (Jacobson, 1963b). A second inductive influence comes from heart mesoderm (Jacobson, 1966). Heterochronic grafts in which the influence of one of the inductors is repeated and that of another reduced or omitted, suggest that influences on prospective lens of endoderm, heart mesoderm and optic vesicle are not qualitatively different (Jacobson, 1966).

Grafts and explants involving prospective lens and non-lens ectoderm suggested that there was also temporal and spatial variation in competence of ectoderm to respond to an inductive signal. In particular, areas that were competent to produce lenses when taken from young embryos were no longer able to form lenses when taken from older embryos (see Liedke, 1955).

Most of the experiments on lens induction from which these various conclusions were drawn suffered from two technical difficulties (reviewed by Saha *et al.,* 1989). Firstly lack of effective donor-host marking meant that contamination of optic vesicles by the fragments of prospective lens could give false -positive results in lens induction assays involving the optic vesicle. Secondly, lens formation was often scored on the presence of ectodermal thickenings, which might be surgery wounds or placodes of other organs. Grainger and co-workers (Henry and Grainger, 1987; Saha *et al.,* 1989; Grainger *et al.,* 1992; reviewed by Grainger, 1992) have repeated classic experiments using unambiguous donor-host marking, and using

crystallin expression to score for lens formation. They found that optic vesicles were unable to induce lenses from flank ectoderm and conversely that lenses could form in the total absence of the optic vesicles. Thus optic vesicles are neither necessary nor sufficient for lens formation (Grainger *et al.,* 1992; Grainger, 1992; but see Karkinen-Jääskeläinen, 1978).

The classical findings of spatio-temporal variation in lens competence could not simply be explained as contamination however (Saha *et al.,* 1989). Recent experiments confirm that the entire gastrula non-neural ectoderm can form lenses, but that ability becomes restricted to the prospective lens area during neurulation (Henry and Grainger, 1987). Moreover, a lens-forming bias is created in the surface ectoderm before it makes contact with optic vesicle (Karkinen-Jääskeläinen, 1978)

The modern model of lens 'induction' is complex (Grainger, 1992). A series of positive lens-forming influences come from endoderm and heart mesoderm and also from the anterior neural plate, a signal which may act in the plane of the tissue (Henry and Grainger, 1990). Ectoderm passes through a series of levels of competence to respond to these inductive signals with competence for a certain signal being acquired and then lost (Servetnick and Grainger, 1991). A lens forming-bias is established in head ectoderm that can be released by permissive signals (Karkinen-Jääskeläinen, 1978), which may be the role of the optic vesicle. Head ectoderm then becomes specified to form lens and can do so if explanted, but this ability also gets narrowed down to the region that actually does form the lens (Barabanov and Fedtsova, 1982). Some negative influences, perhaps the access of neural crest cells to

surface ectoderm, may be involved in the restriction of the various lens-forming tendencies to the region adjacent to the optic vesicle. (Grainger *et al.,* 1992b)

1.1.3 Connections of the visual system and their development.

In mammals, binocular vision attains depth of field perception by integrating the images from the slightly different viewpoints of the two eyes. Growth cones of retinal axons that first enter the optic stalk about E12.5 in the mouse, start to arrive at the chiasm from E13 (Colello and Guillery, 1990,1992). Here axons either cross to the opposite optic tract or enter the optic tract on the same side. Virtually all axons originating in the nasal region of the retina have a crossed axon. Uncrossed axons are generally from the temporal side of the retina (Colello and Guillery, 1990). The optic tracts containing axons from both eyes project to the lateral geniculate nucleus, from where further axons project to the visual cortex. There are complex connections within the visual cortex (Gilbert and Wiesel, 1979) and connections are then made from the visual cortex to the thalamus and to the superior colliculus, the mammalian homologue of the optic tectum of other vertebrates.

The complex wiring of the visual system is partly accomplished during development by the choices made by growth cones, for example at the chiasm, (Colello and Guillery, 1990). Other mechanisms are involved (see Goodman and Shatz, 1993 for a review), particularly in the refinement of connections. Early, inappropriate connections are made (Simon and O'Leary, 1990) with 'misdirected' axons being subsequently eliminated (Inoue *et al.*, 1992).

The best characterised of these refinement mechanisms is in the visual cortex, where input from the two eyes is arranged into alternating columns of cells, the

ocular dominance columns. These are established by an activity-dependent remodeling mechanism, probably involving synaptic competition for limiting quantities of neurotrophins (Cabelli *et al.,* 1995).

1.2 The olfactory system

1.2.1 Structure of the nasal passages.

The nasal passages of higher vertebrates are paired cavities separated medially by a nasal septum (general references, Kahle, 1986; Grays' Anatomy, 1973). The cavities open anteriorly at the nostrils and communicate posteriorly with the nasal part of the pharynx. A series of elevations, the conchae, project into the cavity, greatly increasing its surface area and perhaps serving to create a slower, more turbulent, airflow. Only the more posterior and dorsal regions of the main nasal cavities are lined with a sensory, olfactory epithelium. Elsewhere the lining is a non-sensory, respiratory epithelium. There is also a blind pocket, the vomeronasal organ, which is lined with an epithelium similar to the olfactory epithelium, and is thought to have an additional olfactory function.

The olfactory epithelium has a relatively simple structure mainly consisting of sensory cells, supporting cells and basal cells. In terrestrial vertebrates it is covered by a fine film secreted by small mucous glands, the Bowman's glands, that are scattered throughout the epithelium. The shafts of the sensory cells extend beyond the microvilli-covered surface of the supporting cells into the mucous layer. At the end of the shaft is a bulbous terminus from which a large number of sensory cilia extend into the mucous layer.

The detection of odorants probably required them to be first dissolved in the mucous layer from the air space. In the mucous they can then interact with odorant receptors on the cilia of the sensory cells. In mammals there is a large repertoire of seven-transmembrane domain olfactory receptors encoded by about a thousand

different genes (Buck and Axel, 1991). Many thousands of different smells can thus be identified by the activation of different combinations of these receptors.

Beyond providing a mechanical support, the roles of supporting cells and basal cells have long been unclear. Studies on nerve cell renewal (Graziadei and Monti-Graziadei, 1985) now suggest that basal cells are stem cells that are the direct precursors of neurons. Supporting cells and some cells of the Bowman's gland contain pigment that is important for sense organ function (Allison, 1953).

1.2.2 The structure and function of the olfactory bulbs

In the eye, photoreceptors pass signals via many types of cell to the ganglion cells, which then send an output along the optic nerve to the brain. In contrast, the olfactory sensory cells themselves send axons that project with no intervening synapses to the brain. These axons terminate in the glomerular layer of specialised protuberances of the basal anterior forebrain, the olfactory bulbs. The olfactory bulbs serve as the primary relay stations for olfactory information (Allison, 1953). Their synaptic organisation has been described for the mammals by Shepard (1972).

Olfactory bulbs have a readily apparent layered structure, shown in Figure 1.4. Each olfactory sensory cell projects a single unbranched axon which fasciculate to form the olfactory nerve. At the olfactory bulb, incoming fibres form the olfactory nerve layer, then penetrate to the subjacent glomerular layer and ramify extensively. There are about 1,000 to 3,000 glomeruli in the glomerular layer of mammals, each containing the terminals of many thousands of olfactory axons, together with the dendrites of three types of olfactory bulb neuron; mitral, tufted and periglomerular cells. Recent studies (Vassar *et al.,* 1994) suggest that axons to each bulb from

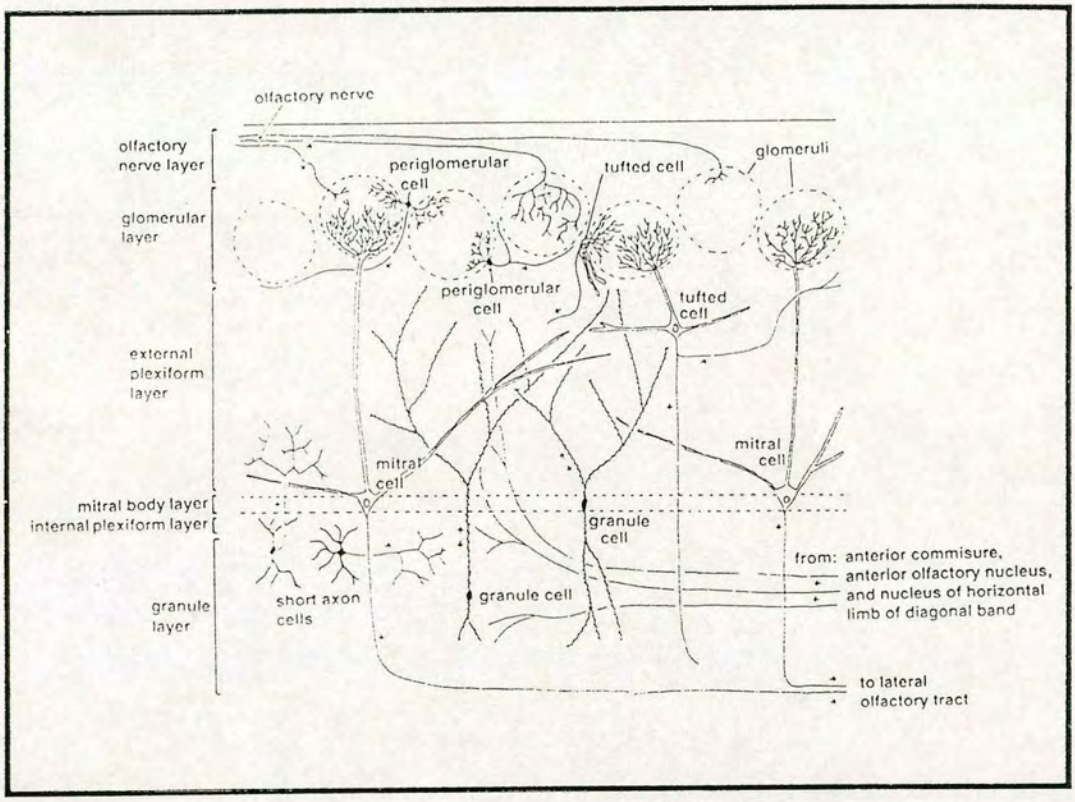

Figure 1.4
Structure of the olfactory bulbs, with morphology and positions of major cell types. Incoming axons of the olfactory nerve arrive in the olfactory nerve layer and penetrate the underlying glomerular layer. In the glomeruli they ramify extensively, making synapses with the dendrites of mitral, periglomerular and tufted cells. Typical laminar positions of nuclei, and processes for each major cell type are shown. Axons are represented by thin lines with arrows to indicate the direction of signals are axons. Granule cells are axonless, indicated by bi-directional arrows. The output of the olfactory bulb is produced by the axons of the mitral cells. Afferent connections from other parts of the brain are indicated. (Based on a figure in Shepard, 1972).

sensory cells expressing a given receptor converge on one, or a few, specific glomeruli, that are stereotypically arranged within the olfactory bulb. Since the number of glomeruli is similar to the number of odorant receptor genes this may suggest that each glomerulus deals with the response to a small number of odorants.

Periglomerular cells probably provide pathways for interglomerular association, but some also make connections with mitral and tufted cells. The mitral cells and some deep tufted cells produce the output of the olfactory bulb, sending axons to the lateral olfactory tract. There are however numerous other connections involved in producing this output. As well as the three types of neuron described above there are also the axon-less granular cells, two types of short axon neuron, and afferent fibres entering the olfactory bulb from the telencephalon.

The vomeronasal organ, sends axons to the accessory olfactory bulb, located on the postero-dorsal region of the main bulbs (Gurdjian, 1925). In the rabbit, the vomeronasal organ contains about one thirtieth of the olfactory receptor cells, and the accessory bulb is a similar fraction of the size of the main bulb. The accessory bulb structure is generally similar to that of the main bulb (Gurdjian, 1925), but with some differences, such as a lack of tufted cells (Allison, 1953)

1.2.3 Secondary and tertiary connections of the olfactory system.

The principal projections from the olfactory bulbs are to the contralateral bulb via the anterior commisure and to the prepiriform cortex (Valverde, 1965). There are two sets of fibres involved, the deep fibres and the lateral olfactory tract axons, which run lateral of the anterior olfactory nucleus. From the anterior olfactory nucleus,

some deep fibres cross the anterior commisure to the contralateral bulb and contralateral anterior olfactory nucleus. Others terminate in the stria terminalis or its continuation, the central amaydaloid nucleus. The stria terminalis (or epistriatum) is a significant subcortical olfactory area. In mammals it is drawn out and attenuated over the large internal capsule. The lateral olfactory tract mainly terminates in the prepiriform cortex, but also partly in the anterior olfactory nucleus, the antero-lateral part of the olfactory tubercle and the amygdala.

Tertiary projections are more difficult to study, particularly in those cases where a secondary olfactory centre contains many non-olfactory fibres, such as the amygdala. This problem is less severe with the prepiriform cortex. Projections from prepiriform cortex run to the ventrolateral portion of the prefronal area. Thus, like other senses, olfaction may be finally represented in the isocortex. Other tertiary projections of the olfactory system include the olfacto -hypothalmic tracts and the olfacto-habenular tracts to the habenular nuclei and hence to the habenulo-peduncular tracts, and so to the motor region of the tegmentum. (Allison, 1953). Some of these tracts are components of the stria medullaris, which at its anterior end is formed by the union of the lateral olfacto-habenular tracts, a connection to the stria terminalis, and the cortico-habenular and septo-habenular tracts (Gurdijan, 1925)

Many of the regions known to receive olfactory input form feedback loops back to the bulbs, involving one, two, or many synapses (Shepard, 1972). Those that form polysynaptic loops, such as that through the nucleus of the horizontal limb of the diagonal band, may be important in combining olfactory and non-olfactory information (Shepard, 1972).

1.2.4 Development of the nose

1.2.4.1 Nasal placode origin of olfactory sensory cells.

The development of the olfactory epithelium in the mouse has been described by Cushieri and Bannister (1975). Like the lens, the nasal cavities are formed by the invagination of ectodermal placodes, the nasal (or olfactory) placodes. These paired placodes, which have a pseudostratified appearance and are separated from the adjacent telencephalon by a wide band of mesenchyme, are the first recognisable sign of nasal development. They are well developed by E9.5 (Kaufman, 1992), their development slightly preceding that of the lens placodes. Around E9.75 the center of the placode starts to invaginate, forming the nasal pits. Studies in the rat indicate that prior to the invagination, the expression of the growth associated protein, GAP-43, is already restricted to a few cell in the center of the nasal placodes (Pellier *et al.,* 1994). Thus there is some pattern present in the placodes even at this early stage.

The GAP-43 positive cells are presumptive olfactory receptor cells (Pellier *et al.,* 1994). In the mouse these start to send out axons which are detectable in the mesenchyme adjacent to the nasal pits at about E10.5 (Cushieri and Bannister, 1975). A day later, the first axons from nasal pit reach the ventromedial aspect of the anterior forebrain. Within the mesenchyme the fascicules of fibres are associated with sheath cells, that are also produced by the nasal placode (Marin-Padilla and Amieva, 1989). In the nasal pit itself, the vomeronasal organ is clearly present as a thickening and outpocketing of the epithelium.

The arrival of the olfactory axons at the brain may induce the formation of the olfactory bulbs (Gong and Shipley, 1995). The relative timings from the mouse of

olfactory nerve penetration of the forebrain and the evagination to form the olfactory bulbs agree with this interpretation (Hinds, 1972a, 1972b; Doucette, 1989).

1.2.4.2 Induction of the nose.

Like the eye, the events leading to the formation of nasal placodes, particularly the role of the forebrain, have been the subject of controversy. Forebrain removal and nasal placode grafting experiments by Bell (1906, 1907), using the frog, *Rana esculenta*, concluded that nasal development was partly independent of the forebrain, from around the time of placode formation, but did not address the question of whether there was a brain-derived placode-forming signal earlier.

There appeared to be evidence however, that the forebrain might induce ectopic nasal placodes. Typical of the experiments of the time, Zwilling (1934) transplanted large pieces of intermediate neurula *Rana pipiens* forebrain into the flank and obtained well developed nasal cavities in ectoderm overlying the graft. Waddington and Cohen (1936) examined the 'postgeneration' phenomena of chick embryos recovering from surgical removal of one side of the head, and found that they would sometimes form nasal placodes on the operated side. From examining these cases they suggested that reforming brain was inducing a nasal placode in new overlying ectoderm.

The grafting experiments suffered from the same lack or host/donor marking that led to lens formation being misinterpreted as de-novo induction. Zwilling (1940) made use of xenografts where there were differences in cell size, nuclear size and pigmentation betweeen *Rana pipiens* donors and *Amblystoma* or axolotl hosts. An inductive signal from neural tissue was expected to act prior to placode formation,

but neural tissue from such stages was unable to convincingly induce ectopic noses in similarly aged or younger hosts.

An early lateral signal from the presumptive neural forebrain remained a possibility however, as was induction by the archenteron roof (Zwilling, 1940). This latter effect was not specific to the nose, but those transplants that would self-differentiate into nose generally included archenteron roof. Medial or medio-lateral portions of anterior archenteron roof were found to induce small ectopic heads complete with brains, ears, olfactory organs and suckers. In one such case there was no brain tissue in the induced head implying that the nose and ear could be induced without brain development.

Jacobson (1963a,b,c), performed a series of explant and transplant experiments which addressed the question of determination and positioning of the nose. At the open neural plate stage of amphibian embryos the mesoderm is essentially below the neural plate, thus the presumptive nasal region is underlain instead by pharangeal wall endoderm (Jacobson, 1963a). In the newt (*Taricha torosa*) all the factors required for positioning the nose are present and active at this stage (stage 15). The most important factor for nose development in these explant experiments was anterior endoderm, although some noses would form if the explant contained anterior neural plate and neural folds. Similar results were obtained on manipulated embryos (Jacobson, 1963b,c). Together the results suggest that either underlying endoderm or anterior neural plate and neural folds can induce nose formation, but normally they act together. The endoderm is of primary importance

and acts at the late gastrula or neurula stages. The influence of the neural plate is somewhat later, and could act in the plane of the epithelium through the neural folds.

1.3 Development and patterning of the CNS:

Reasons for highlighting patterning and fate determination.

The development of mammalian central nervous system (CNS) occupies extended period from gastrulation through to the birth and continues postnatally. Given this time-scale and the exquisite complexity of the final structure the potential exists for a vast range of processes to be involved in CNS development.

The following sections are intended to give an overview of CNS development but two further considerations have guided the choice of topics selected. Firstly this study has revealed abnormal morphogenesis of the early *Sey/Sey* forebrain, thus some prominence will be given to events and processes that may be disrupted. Secondly particular attention will be paid to the mechanisms of patterning of the nervous system and the control of cell fate. There are a number of reasons for these latter choices. 1) *Pax6* expression is clearly spatially restricted in the developing nervous system from shortly after it first appears (Walther and Gruss, 1991; this study). This suggests that PAX6 is probably acting in subsets of CNS cells rather than throughout the CNS. Thus the differences between cells in different parts of the CNS, and how these differences are acquired, may be of special interest. 2) Domains of *Pax6* expression may reveal underlying patterns in the nervous system that precede morphologically recognisable patterns, making it worthwhile to consider the extent of patterning at early stages. 3) The *Small eye* (Sey^{Neu}) mutation disrupts cerebral cortex development in a manner consistent with a role for *Pax6* in cells switching from proliferation in the germinative epithelium to migration and differentiation to form the cortical plate (Schmahl et el., 1993). The layered structure of the normal

cortical plate depends in part upon cell fates acquired in the germinative epithelium. (McConnell, 1988; McConnell and Kaznowski, 1991). 4) Related genes in other species, such as the *Drosophila* genes *gooseberry* and *pox-neuro*, appear to specify the fate of specific cells in the development of the nervous system (Patel *et al.,* 1989; Dambly-Chaudiere, *et al.,* 1992; Ghysen and Dambly-Chaudiere, 1993; Nottebohm *et al.,* 1992,1994; Zhang *et al.,* 1994).

As with the development of the eye and nose, the stages examined in this study are entirely prenatal, and the introductory chapters reflect this.

1.3.1 Induction and early regionalisation of neural tissue.

In amphibia the dorsal lip of the blastopore has an organiser activity and recruits surrounding cells to form the antero-posterior axis (reviewed by Spemann 1938). This involves the induction of neural tissue from ectoderm, and is thus of considerable interest for investigating the origins of the neural tissue. Tissue culture and recombination experiments suggest that *Xenopus* ectoderm first becomes specified to make neural tissue during gastrulation. Dorsal mesoderm from the organiser can provide the required inductive signal for this specification, with prolonged contact with the organiser possibly being required for complete expression of neural characteristics. It seems likely that similar processes also occur in chick and mouse, where the node has been found to have organiser activity. (Waddington, 1932; Beddington, 1994).

There is evidence for the existence of multiple neural inducing activities, acting either directly or through the induction of mesoderm (reviewed by Harland, 1994). Observations that support this idea include; 1) Some patterning occurs

coincident with neural induction (Storey *et al.,* 1992). 2) The same ectoderm will form neural tissue with characteristics of anterior or posterior CNS depending upon the A-P axis levels from which the neural inducers was isolated (Leussink, 1970; Sharpe and Gurdon, 1990). 3) Candidate neural inducing molecules do not induce both anterior and posterior neural types equally. Noggin, for example, induces the expression of only anterior neural markers (Lamb *et al.,* 1993).

Genetic evidence for separate organisers comes from the targeted mutagenesis of the mouse *Lim1* gene, implicated in organiser function. Homozygous mutants for the *Lim1* gene fail to make head structures anterior of rhombomere 3, but have largely normal development of the body, indicating the existence of a molecularly distinct head organiser (Shawlot and Behringer, 1995).

Presumptive notochord, a major fate of dorsal blastopore lip cells, may be the source of some of the regionalising signals associated with the node or dorsal blastopore lip. Once presumptive anterior notochord cells leave the node, the node is no longer capable of inducing diencephalic or mesencephalic structures (Storey *et al.,* 1992).

The mechanisms by which brain and posterior CNS are produced may be somewhat different. It is possible that the node is not linked to posterior neural induction, as in older chick embryos, in which posterior neural structures are being formed, the neural plate extends posteriorly some distance behind the node. One explanation argues for homeogenetic induction, where posterior neural structures are induced by horizontal signals in the plane of the epithelium from previously induced epiblast (reviewed by Jacobson and Sater, 1988; Ruiz I Altaba, 1992). One

alternative explanation suggests that a small primordium of trunk CNS is induced at the same time as the head and subsequently elongates out of step with the node movements.

Fate mapping in the mouse (Quinlan *et al.,* 1995) suggests that some regionalisation of the nervous system occurs well before neurulation, and at about the time during which neural fates are being acquired. Almost the entire precursor population of the neural tube is contained within the distal cap region of the epiblast, and there is already some regionalisation within this population.

1.3.2 The neural plate and mechanisms of neurulation.

As discussed above, the nervous system appears to become specified during gastrulation. The result is the neural plate in which an indentation appears, the neural groove. In the mouse the neural groove has double origins producing a rostral and a caudal groove (Källén, 1952). The neural plate then roles up longitudinally, centered on the neural groove, in a process termed primary neurulation, to form a tube-like structure which expands at the brain end. Secondary neurulation, a distinct process involved in the formation of the most caudal neural tube, will not be discussed here.

Neurulation is a complex process, probably involving both mechanisms intrinsic to and extrinsic to the neural plate (see Schoenwolf and Smith, 1990 for a review). Moreover there are many examples where morphological changes and processes involved are not uniform along the antero-posterior axis, including the initial directions of folding of the neural plate, the directions of mitotic division planes, the relative influence of underlying mesenchyme, and of forces extrinsic to the neural plate (Moriss-Kay, 1981; Jacobson and Tam, 1982; Chen and Behringer,

1995). The variation in the direction of mitotic planes is of particular note, since cell division in midbrain and hindbrain is parallel to the axis of the embryo, but does not result in a local elongation. Instead there is probably a net movements of cells into the forebrain (Moriss-Kay, 1981).

All neuroepithelial cells undergo changes in shape during neurulation, related to interkinetic migration, a movement of the nucleus of the cell between cell divisions (Schoenwolf and Smith, 1990). Co-ordinated changes in cell shape, such as the production of wedge shaped cells and cell elongation, do however appear important for the shaping and bending of the neural plate during neurulation (Jacobson , 1981; Shoenwolf and Smith, 1990).

At the regions of maximum curvature, or hinge points, cell shape changes occur only when neural plate has been anchored to adjacent tissues, the notochord or surface ectoderm respectively (Schoenwolf and Smith, 1990). Influences from other tissues and extracellular matrix might thus also be important in shaping and bending the neural plate.

There are some notable differences between neurulation in differences species, (illustrated by Morris-Kay, 1981). Amphibians maintain a fairly constant size and cell number of the neural plate during neurulation, thus cell movement (Jacobson, 1981), attributed principally to intercalation of cells, seems especially important in Xenopus, first in narrowing of the neural plate and then lengthening it (Keller *et al.*, 1992). Chick and mouse neural plates are effectively one cell thick throughout neurulation, and so cell movement, although it occurs in chick

neurulation (Schoenwolf, 1991), may be a less significant mechanism in these species.

Mouse embryo neural plates, as well as rolling up along the longitudinal axis, form flexures at points along the axis, starting with the primary cranial flexure which appears well before neural tube formation (Jacobson and Tam, 1982). These flexures are important for establishing the three dimensional arrangement of brain structures and may exert a mechanical influence on a number of tissues. Bends in the embryonic axis may assist in the early closure of the neural fold in the cervical region and prosencephalon and mechanically inhibit closure in the midbrain region (Morris-Kay, 1981; Jacobson and Tam, 1982). Moreover, the rostral tip of notochord shifts in position in relation to the brain, probably due to strong brain flexures (Bergquist and Källén, 1954).

1.3.3 Major subdivisions of the CNS are present early in development.

The adult CNS can be divided into units along the axis, the forebrain, midbrain, cerebellum, hindbrain and spinal cord. These units essentially develop from earlier divisions of the neural tube into vesicles. Thus the forebrain (telencephalon and diencephalon) develops from prosencephalon, midbrain from mesencephalon, cerebellum from metencephalon and hindbrain from rhombencephalon. The correspondence between brain vesicles and later structures is not precise however, and in particular there is a contribution to the rostral cerebellum from the cells in the caudal mesencephalon (see Hallonet *et al.*, 1990).

Longitudinally the spinal cord possesses thin plates dorsally and ventrally, the roof plate and floor plate respectively. The thicker lateral walls are divided into an

alar and a basal plate separated by the sulcus limitans. These longitudinal domains continue rostrally into the brain, although not all levels of the neural axis posses the full complement of plates. Thus the mesencephalon lacks the thin roof plate of more posterior regions, and rostral of the notochord, in the prosencephalon, there is no true floor plate.

Signs of the later division of the brain can be detected well before neural tube closure. In the mouse, the prosencephalon, mesencephalon and metencephalon are discernible in one somite embryos as dorsal bulges separated by constrictions of the neural plate, and these divisions may even be preshadowed in late presomitic embryos (Jacobson and Tam, 1982).

In *Xenopus*, fate mapping experiments (Jacobson and Klein, 1985) suggest that some major subdivisions of the brain are part of polyclonal compartments. In particular there is little cell mingling between dorsal and ventral regions, even anterior of the sulcus limitans. Moreover the populations that form hindbrain and spinal cord are largely, but not entirely, separate from those that form the forebrain and midbrain.

1.3.4 Early commitment to regional identity

The commitment of domains of neuroepithelium to their region-specific fate has been investigated by heterospecific grafts between 2 day old (about 7-15 somite) chick and quail embryos by Alvarado-Mallart *et al.,* (1990). Some domains such as the caudal metencephalon and caudal mesencephalon were found to be essentially committed to cerebellar and midbrain fates respectively. Other domains were still pluripotent and the cytoarchitecture they adopted could be influenced by their

environment. Thus prospective caudal diencephalon of the host could form a lamina structure characteristic of midbrain if, by grafting, it became surrounded by mesencephalon. Rostro-caudal inversions centered on the mesencephalon, demonstrated that a polarizing activity is present in the prospective ithmocerebellar region, probably located in the caudal-most part of the mesencephalon (Martinez and Alvarado-Mallart, 1990; Marin and Puelles, 1994). This polarizing zone is sufficient to organize a set of midbrain structures from prospective midbrain or caudal diencephalon.

In the mouse there are region-specific differences in potential of the neural ectoderm that become apparent at comparable developmental time-scales to the chick studies described above. Choroid plexus normally appears *in vivo* from around E13.5 in the lateral and fourth ventricles (Kaufman, 1992). Thomas and Dziadek, (1993) studied the capacity to from choroid plexus like cells *in vitro* from E8.5 or E9.5 tissue. At E8.5 extensive regions including mesencephalon would form choroid plexus in culture, but by E9.5 this capacity was closely related to the areas that actually would form choroid plexus *in vivo*.

1.3.5 Dorsal-ventral patterning of the neural tube.

The dorso-ventral divisions of the neural tube are best illustrated by the spinal cord which is formed from the full complement of floor plate and roof plate, alar and basal plates. The lateral walls of the spinal cord are formed from the alar plate dorsally and basal plate ventrally. There is a functional distincion between these plates with sulcus limitans at their junction dividing the spinal cord into a dorsal, sensory region and a ventral, motor region. There are further subdivisions in the cell

types produced at different dorso-ventral positions (see Ramon y Cajal, 1960). In particular the the regions immediately dorsal and ventral of the sulcus limitans are considered as viscero-sensory and viscero-motor domains. The dorso-ventrally restricted expression of genes such as the *Xenopus* homeobox gene *XeNK-2* and the notch ligand *Jagged* (Saha *et al.,* 1993; Lindsell *et al.,* 1995), do not correspond to any morphologically defined boundaries or functionally defined regions, revealing that still more dorsal-ventral pattern is present in the neural tube.

The floor plate of the neural tube is induced by a signal from the underlying mesoderm-derived notochord, and in turn influences the development of dorsal-ventral pattern of the lateral neural tube, (reviewed by Placzek *et al.,* 1991). There is an interplay between signals from notochord or floor plate and factors expressed dorsally in the neural tube. In particular the dorsally expressed gene, *dorsalin-1*, encoding a TGFβ -like molecule, promotes neural crest differentiation, inhibits the formation of motor neurons and is repressed by notochord signals (Basler *et al.,* 1993).

Notochord induction of floor plate is suggested by lack of floor plate in various notochord deficiencies produced by UV treating *Xenopus* embryos (Clarke *et al.,* 1990) and surgically removing Hensen's node in the chick (Hirano *et al.,* 1991) as well as analysis of the notochord deficient mutants, zebrafish *cyclops* (Hatta *et al.,* 1991), and mouse *Danforth's short-tail* (*Sd*) (Bovolenta and Dodd, 1991). More direct evidence comes from ectopic notochords (Watterson *et al.,* 1965; van Straaten *et al.,* 1988) and co-culture experiments assaying for the floor plate property of producing a chemoattractant for commisural axons (Placzek *et al.,* 1990). Such

experiments also demonstrated the existence of a contact-dependent homeogenetic signal from floor plate inducing further floor plate (Hatta *et al.,* 1991; Placzek *et al.,* 1991; Placzek *et al.,* 1993). The non-autonomous action of the *cyclops* mutation in the forebrain (Hatta *et al.,* 1994) suggests that signaling from the ventral midline also occurs prechordally, i.e. where there is no notochord.

The most severe disruption of dorso-ventral pattern in the neural tube of notochordless animals stems not from absence of notochord, but from the lack of floor plate (Hirano *et al.,* 1991). In the spinal-chord, floor plate produces a diffusible factor which can induce motor neurons (Yamada *et al.,* 1993) and inhibit formation of dorsal structures.

In the midbrain there is a contact-mediated induction of dopaminergic neurons by floor plate (Hynes *et al.,* 1995). All floor plate is capable of providing this signal but only mesencephalon can respond to it, suggesting that in this case some antero-posterior patterning precedes the dorso-ventral induction events (Hynes *et al.,* 1995). A number of genes, including Pax genes (Stoykova and Gruss, 1994) and homeobox genes (Saha *et al.,* 1993) are expressed in domains that are restricted both antero-posteriorly and dorso-ventrally, so the same molecules could be involved in patterning both axes.

1.3.6 Neuromeres and proneuromeres: transient subdivisions of the brain

Further recognisable subdivisions of the brain exist. Comparative analysis of adults and embryonic brains from many vertebrates (reviewed by Kuhlenbeck, 1973), suggested that these are ancient and important features of the overall organsiation of the brain.

Along the axis, successive waves of bulges and constrictions appear and then disappear, transiently forming transverse domains, first forming the proneuromeres which subdivide to form the neuromeres (reviewed by Berquist and Källén, 1954). Thus in the mouse, two neuromeres form from the single mesencephalon and the three rhombencephalic proneuromeres form seven rhombomeres (Källén and Lindskog, 1953). Subdivision of the prosencephalon into neuromeric units is less clear cut.

1.3.7 Transverse domains in the developing forebrain.

Following the closure of the neural tube in the mouse, the prosencephalon undergoes considerable expansion, particularly an elongation. (Jacobson and Tam, 1982). In this expansion, paired evaginations of the rostral prosencephalon form the telencephalic vesicles. The caudal prosencephalon forms the diencephalon, which becomes the thalamus. The timing of these events is interesting, as Jacobson and co-workers showed, the cerebrospinal fluid pressure that develops after neural tube closure is important for the expansion and overall shape of the brain (Desmond and Jacobson, 1977; Jacobson 1981). In addition to the mechanisms discussed for bending neuroepithelium during neurulation, differential resistance to the cerebrospinal fluid pressure-driven expansion could be important in changes in brain shape (Jacobson, 1981).

The diencephalon is divided into neuromeric units (Figdor and Stern, 1993) with similar properties to rhombomeres, as discussed below. There is some doubt as to how many such units there are, and what later structures they correspond to (see Puelles *et al.*, 1987). Most authors agree, however, that there are at least three units

(Figure 1.5A), a synencephalon or pretectum area (which may be two units) at the posterior of the diencephalon, the presumptive dorsal thalmus (posterior parencephalon) and the presumptive ventral thalamus, (which may or may not be a separate neuromere from the hypothalamus). In addition the epithalamus is recognised as a separate domain, but it does not form a complete transverse ring as it is present only dorsally (Puelles *et al.*, 1987; Lakke *et al.*, 1988; Figdor and Stern. 1993; Puelles and Rubenstein, 1993).

In the telencephalon, the complex topology means that simple longitudinal and transverse domains are not readily apparent. Comparative neuroanatomy (see Bergquist and Källén, 1954) suggests that the telencephalon itself is a secondary evagination and may not be a transverse unit in the same sense as the proneuromeres and neuromeres. Bergquist and Källén, (1954) also suggest that the optic vesicle may hide other important boundaries. Despite such difficulties, a number of both basal and dorsal subdivisions can be described (Kuhlenbeck, 1973). These are shown in Figure 1.5 B for the mouse (from an original figure by Ian Smart).

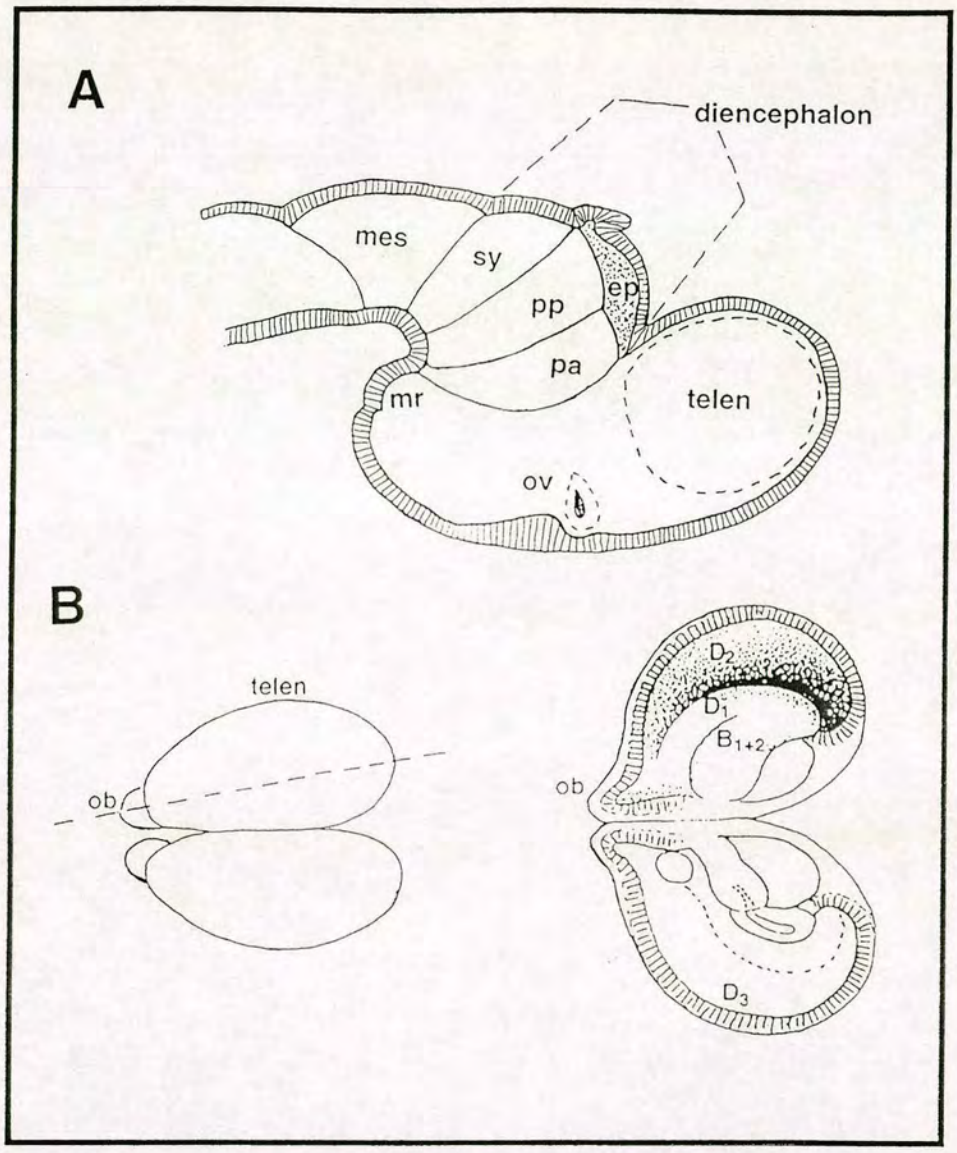

Figure 1.5
Morphological subdivisions of the forebrain. (A) Divisions of the diencephalon based on the model of Puelles *et al.*, (1987). The diencephalon is divided into three units, the synencephalon (sy), posterior parencephalon (pp) and anterior parencephalon (pa). The synencephalon is equivalent to the pretectal area, and may be two compartments (Figdor and Stern, 1993). The posterior parencephalon is equivalent to the prospective dorsal thalamus, and is a compartment. The anterior parencephalon is in the region of the prospective ventral thalamus, and dorsal of the prospective hypothalamus. The epithalamus (ep) is recognised as an additional subdivision of the diencephalon. telen, telencephalon; mes, mesencephalon; mr mammilary recess; ov optic vesicle. (B). Some morphological domains of the telencephalon, based on an original figure by Ian Smart, with the notation of Kuhlenbeck (1973). A dissection of the telencephalic lobes from a mouse embryo at E14.5 reveals lateral and medial ventricular surfaces on which dorsal (D_1, D_2 and D_3) and basal (B_{1+2}) subdivisions are indicated. telen, telencephalon; ob, olfactory bulbs.

1.3.8 Longitudinal and transverse domains are proliferation and migration centers.

The neural plate and early neural tube are relatively undifferentiated but rapidly adopt a pseudostratified epithelium structure (see Watterson, 1965 for a review). There is no specialised proliferative layer, rather cell bodies undergo interkinetic migration, moving during S phase towards the ventricular surface where most division takes place, and with daughter cell bodies migrating away again. This pattern of interkinetic movements continues throughout development, and is an important feature of the developing cerebral cortex.

The main longitudinal and transverse domains of the brain, including the rhombomeres, are proliferation and migration centers (Källén, 1965). Both proliferation and subsequent migration is higher within these domains than at their boundaries, and there are differences in both properties between domains (reviewed by Bergquist and Källén, 1954; Layer and Alber, 1990). These regulated patterns of proliferation are important for the morphology of the neural tube, particularly that of the neuromeres (Källén 1962, Källén 1965). In the last few years it has been recognised however that neuromeres are not only migration and proliferation centers, but are also compartments.

1.3.9 Properties of neuromeres and their boundaries

The neuromeres of rhombencephalon and diencephalon form polyclonal compartments between which there is little cell mixing (Fraser *et al.,* 1990; Figdor and Stern, 1993; but see Birgbauer and Fraser, 1994). Moreover cells from two odd -

numbered or two even-numbered rhombomeres generally mix, but with juxtaposition of cells from an odd and an even numbered rhombomere there is little cell mixing and a new rhombomere boundary may be formed (Guthrie and Lumsden, 1991; Guthrie *et al.,* 1993). These results suggest the distinction between adjacent compartments is established or maintained by homophilic recognition, perhaps by differential expression of adhesion molecules. Two unit periodicity in the hindbrain (reviewed by Lumsden and Guthrie, 1991) is also apparent in exits of cranial nerves (Lumsden and Keynes, 1989) in neural crest emigration (Lumsden *et al.,* 1991), and in patterns of gene expression (Kuratani, 1991). As two neuromeres often correspond to one proneuromere (Källén and Lindskog, 1953) this two unit periodicity lends credence to idea that proneuromeres are important divisions.

The rhombomere boundaries themselves may be distinct from the body of the neuromere. Cells in the boundary regions are packed differently from those in the body of rhombomeres (Lumsden and Keynes, 1989; Heyman *et al.,* 1993), have lower rates of proliferation mentioned above (Layer *et al.,* 1990), and differentially express other molecular markers, including *Pax6* (Lumsden and Keynes, 1989; Layer *et al.,* 1990; Quiling Xu, personal communication).

As the brain is being divided into units, the first signs of neuronal differentiation are also apparent. In the chick hindbrain, where neurogenesis follows a two segment repeat, this pattern of axon formation is apparent at stage 13, shortly after the formation of the rhombomeres from stages 9 to 12 (Lumsden and Keynes, 1989). The next sections will deal with how cells acquire a particular fate, and how correct axonal connections are made.

1.3.10 Separate precursor populations for major cell types in the CNS.

Retroviral labeling experiments in the developing cerebral cortex generally give clones of a single cell type (Luskin *et al.,* 1988; Parnevelas *et al.,* 1992; Grove *et al.,* 1993; reviewed by Guthrie, 1992). Together these results suggest up to seven separate precursor-cell types (Grove *et al.,* 1993). The lineage restriction between neurons and glia is made before E12 in the mouse (Luskin *et al.,* 1988), but cell labeling experiments in amphibians suggest that the restriction to glia or neuron has not occurred in the neural plate (Soula *et al.,* 1993). Glial cells appear in cultures of dissociated embryonic brains in line with their appearance *in vivo* (Abney *et al.,* 1981). This suggests that glial precursors follow a programme, with a biological clock rather than positional cues governing glial cell production. Thus major cell - type lineages are separated early and some of their subsequent behaviour may not depend on their surroundings.

1.3.11 Extrinsic influences on cell fate.

The fate of many cells in the nervous system can however be controlled by their environment, either by direct contact with the surrounding structures or mediated by diffusible molecules. Examples where the induced change of fate requires contact include the induction of dopaminogenic neurons in ventral midbrain by the floor plate (Hynes *et al.,* 1995). The composition of the extracellular matrix may have an important influence on cell fate. Consistent with this, Reh *et al.,* (1987), found that the presence of laminin greatly increases the rate of in transdifferentiation from *Rana* tadpole retinal pigment epithelium (RPE) to neural retina.

Many diffusible molecules may influence the fate of cells in the nervous system. One good example is the induction of motorneurons in the developing spinal cord by a diffusible signal from the notochord (Yamada *et al.,* 1993), probably the hedgehog homologue, vhh (Roelink *et al.,* 1994). More examples can be found in the review by Jessell and Melton, (1992).

1.3.12 Lamina fate in the cerebral cortex.

The mammalian neocortex develops in a stereotypic manner the deepest layers of the cortical plate forming first. In contrast to the lineage dependence of the cell-types, the level that a neuron migrates to is influenced by its environment (McConnell, 1988). This laminar fate is acquired not on arrival at the at the cortical plate, but by the precursor cell before its final mitosis (McConnell and Kaznowski, 1991). How the surroundings influence the precursor cell is unknown, but there is a great deal of communication between cells in the developing cortex. For example, columnar clusters of neuroblasts are coupled together by gap junctions (Lo Turco and Kreigstein, 1991).

1.3.13 Area patterning of the cerebral cortex.

The cerebral cortex is divided into many functionally separate domains that deal with distinct tasks. There are several suggested mechanisms by which functionally distinct areas of cerebral cortex might come to be established and patterned (reviewed by Grove, 1992). Rakic (1988) suggested a radial unit model, pointing out that if areas of the proliferative ventricular zone were already somehow distinct, then the pattern could be transferred by the columns of migrating cells to

pattern the cortical plate. Since then a number of regulatory genes have been described expressed in subsets of the CNS ventricular zone, making a early pattern more plausible (Price *et al.,* 1992; Tao *et al.,* 1992; Simeone *et al.,* 1992; Simeone *et al.,* 1994). That commitment to form one region of cortex or another may indeed be acquired early is suggested by studies of commitment to express the limbic system-specific marker, LAMP (Barbe and Levitt, 1991; Ferri and Levitt, 1993).

A number of studies have recently shown that some cells migrate tangentially over significant distances in the developing and adult cortex (Lois and Alvarez-Buylla, 1994; Tan and Breen, 1993; Fishell *et al.,* 1993; Walsh and Cepko, 1993; O'Rouke *et al.,* 1992). In particular, lineage studies (Walsh and Cepko 1992) have revealed cells from the same clone in functionally distinct areas, for example the visual cortex, somatosensory cortex and motor cortex (reviewed by Guthrie, 1992) suggesting that a pattern acquired at the ventricular zone might get scrambled by subsequent cell movements. The results of some heterotopic grafts are also at odds with an early acquisition of area pattern and suggest that functional identity may be imposed on an relatively indifferent 'protocortex' by innervation, which may or may not require neuronal activity. In grafts from the visual cortex into somatosensory cortex, the pattern elements appropriate for the host site are imposed on the graft (Schlagger and O'Leary, 1991). Whilst recognisable pattern might be imposed on these grafts ,they do not function as well as homotopic grafts when the host animal is assessed for somatosensory perception or motor tasks (reviewed by Grove, 1992). Overall it thus appears that some regional identity is acquired early and cannot be

subsequently overwritten, but there is still considerable plasticity with aspects of pattern being produced in response to later local cues, probably innervation.

1.3.14 Early axonogenesis: problems of pathfinding

Precise connections must be made between distant parts of the CNS and between the CNS and peripheral tissues such as sense organs and muscles. The mechanisms thought to be involved have been recently reviewed by Goodman and Shatz, (1993). To a limited extent connections can be fine-tuned by producing many initial connections then reinforcing some by activity and eliminating the others. This still leaves a considerable problem of how axon growth cones navigate to their targets to make approximate initial connections.

There are usually considered to be two major ways in which growth cones may navigate. Firstly by following gradients of diffusible chemo-attractant molecules produced by the target (reviewed by Travis, 1994). Secondly by recognising molecules expressed along the pathway. Repulsion, either from particular tissues or by substrates preventing growth cone migration over them, may be considered as variations on these two themes.

Commissural axons produced by dorsal spinal cord respond to a long range chemo-attractant, produced by the developing floor plate (Tessier-Lavigne *et al.*, 1988). A pair of genes, *netrin-1* and *netrin-2*, encode factors that have a long-range axon outgrowth promoting activity and are chemotropic factors (Kennedy *et al.*, 1994; Serafini *et al.*, 1994). Together *netrin-1* expressed in the developing floor plate and a *netrin-2* in the spinal cord probably act both to promote the initial ventral growth of commisural axons and then the turning of growth cones ventrally towards

the floor plate (Kennedy *et al.,* 1994). The netrin-1 molecule has also has a chemo-repulsive effect on axons that normally grow dorsally away from the floor plate, showing that the same molecules can be involved in attraction and repulsion (Colamario and Tessier-Lavigne, 1995).

The role of contact and adhesive specificities in axon targeting has been reviewed by Hynes and Lander, (1992). Extracellular matrix molecules have axon-outgrowth promoting activities. Laminin also has a axon-guidance role (Gunderson, 1987), and is found specifically on astro-glia along certain axon pathways, such as in the optic nerve (Leise and Silver, 1988). Axons fasciculate forming bundles, thus later axons can navigate much of their route by following earlier axons. Adhesion molecules also have axon-outgrowth promoting properties, but are also expressed on axons themselves and may be important in fasciculation, and thus in the choice of route followed (Hynes and Lander, 1992). Contact-mediated inhibitory effects on growth cones are also known or suspected. Tenescin has anti-adhesive properties. Its effect on growth-cones is unclear, but it is expressed at boundaries through which axons do not cross. (Stenideler at al, 1989).

1.4 Questions in the development of eyes, nose and brain.

Studies in developmental biology almost always raise far more questions than they solve. Work that suggests that the polarity of the lens is determined by the retina (Yamamoto, (1976) leads to the question of what the signal might be. Finding a distinct organiser for the head (Shawlot and Behringer, 1995), raises the questions of how many other organisers there might be and whether or not they will involve similar or completely unrelated molecules. From finding one regulatory gene important for a process it is natural to then ask what the targets of this gene might be, how it might itself be regulated, and whether similar genes are acting in similar processes in other tissues or other species. Progress is made however by each finding transforming general questions, such as "How is this structure put together?", into more and more specific ones. Examples of some of the current questions in the development of the eyes, nose and brain have been raised in the preceding introductory sections but are reiterated here.

In current studies of eye development it is still of vital interest to understand how the region that will form the lens is defined, and how its position is co-ordinated with the position of the optic cup. For example, since much of the head surface ectoderm can form lens early in development (Karkinen-Jääskeläinen, 1978; Barabanov and Fedtsova, 1982) what causes this ability to be lost? The influence of the lens on the developing retina is also unclear (Coulombre and Coulombre, 1964). Another important area of interest is in how the complex structure of the retina is formed. Lineage studies make it almost certain that cell type fate in the developing retina depends upon local environmental cues (Turner and Cepko, 1987; Holt *et al.*,

1988; Turner *et al.,* 1990), but there is little evidence as yet to suggest what these cues might be.

The process by which nasal development is initiated has been the subject of controversy, just as lens induction has, but nasal development has not received the same attention. Thus it is still unclear what factors are important and when they act, particularly concerning the influence of the brain on the nose. The mesenchyme rather than the epithelium is known to control the morphogenesis of facial processes (Richman and Tickle, 1989). Mesenchyme underlying the nasal placodes is a source of retinoids (LaMantia *et al.,* 1993) but it is not known what influence these cells might have on nasal cavity formation. Within the ectoderm that forms the nasal cavities there are problems of patterning typical of those throughout the organism. There is already some differential gene expression within the placode (Pellier *et al.,* 1994) and the expression of odorant receptor genes shows that ultimately, patterning within the nasal cavities is extremely complex (Ngai *et al.,* 1993; Ressler *et al.,* 1993), but there is as yet little clue as to how any of this patterning is established. Similarly, the question of how neuronal circuitry is generated in the olfactory system raises issues typical of those that apply throughout the CNS. Positioning tracts of axons may involve a combination of influences. Pini (1993) found that the olfactory bulb cells responded to a diffusible chemo-repulsive activity produced from the midline, such that the olfactory tracts were produced laterally and not medially, but what other factors are involved? Moreover, how is the remarkable topographic specificity (Vassar *et al.,* 1994) of projections from the olfactory epithelium to the glomeruli of the olfactory bulbs established ?

In the studies of the early brain there is an ongoing debate as to what the important subdivisions of the brain are (Puelles *et al.*, 1987). Regions of the brain appear to be divided into neuromeric compartments (Fraser *et al.*, 1990; Figdor and Stern, 1993), but do neuromeres in the forebrain have the same properties as the rhombomeres of the hindbrain? How are the differences between units established? The boundaries of the units may have some distinct properties (Lumsden and Keynes, 1989; Layer *et al.,* 1990; Heyman *et al.,* 1993). What other properties might boundaries have, and how important are they? Similar questions can be asked about the longitudinal domains. In particular it is of interest to know how they relate to the transverse domains, and whether the same molecules are involved in establishing both patterns (Saha *et al.,* 1993).

Some of the developmental questions that may be raised are common to two or all of the systems being considered in this thesis. Asking about the processes that drive optic cup formation for example may be similar to asking how the complex morphology of the brain is produced from a relatively simple neural plate (Brady and Hilfer, 1982; Yang and Hilfer, 1982; Svoba and O'Shea, 1987; Schoenwolf and Smith, 1990). Similarly, although lineage may be more important for cell-type fate in the brain than in the retina (Luskin *et al.,* 1988; Parnevelas *et al.,* 1992; Grove *et al.,* 1993; reviewed by Guthrie, 1992), lamina position is acquired by cells in the cerebral cortex in response to local cues (McConnell and Kaznowski, 1991), just as fates are acquired around the time of division of the precursor cells in the developing retina (Waid and McLoon, 1995).

Relationships between systems such as eye and nasal development could be revealed by common features of gene expression in both systems, or by pleiotroptic mutations, such as *Small eye* (Hogan *et al.,* 1986) that affect the development of both structures. The following sections discuss the information that may be obtained from such approaches, and the way in which genes important in a developmental processes may be isolated.

1.5 Molecular and genetic approaches to developmental problems.

1.5.1 Information provided by gene expression patterns.

Important biological questions, including some of those outlined above, can partly be addressed by an analysis of gene expression patterns. For a specific gene being studied it may be possible to identify likely roles for the gene, or exclude some potential roles, on the basis of the pattern of expression. The way in which that gene is regulated and its possible relationship to other genes may also be suggested from its expression.

More generally, considerable information about the organisation of the organism being studied can be obtained by the same experiments. It is of interest to know, for example, when two tissues are distinct at a molecular level, and how this difference relates to differences in morphology and to the commitment of cells to specific fate. Similarly there may be many more boundaries obeyed by cells than are apparent using traditional histological techniques. Thus, where boundaries of gene expression are not recognised morphological boundaries, they may reveal complexity within the organism that was not otherwise apparent.

Complex gene expression patterns can also reveal underlying relationships between different tissue in the organism, complementing such studies as manipulation of embryos and tissue culture experiments. For example, diverse sites of epithelium-mesenchyme interactions such as the nasal cavities, the developing tooth germs and limb buds, all express *Msx*-genes (Hill *et al.,* 1989; Mackenzie *et al.*, 1991a, 1991b), which suggests some developmental processes at these sites are similar at a molecular level.

1.5.2 Mutant phenotypes provide insight into normal development.

Underlying relationships between different structures can also be revealed by the phenotypes of pleiotropic mutants. Detailed information about the function of a gene can be revealed by mutant phenotypes since it is possible to determine not only the location of a defect, but also manner in which development is disrupted there. There may be redundancy within the gene expression pattern such that a gene is not important everywhere it is expressed. In particular a gene may not be important at the time it is first expressed.

The information provided by a mutant phenotype and by a gene expression pattern can reinforce and complement each other. A severe early phenotype may mask roles of the gene later in development, that may be revealed by expression information. Similarly expression patterns may point the way to subtle defects that might not otherwise be detected. Expression information can also be used to identify some secondary defects within a phenotype, if they are in non-expressing tissues. In this way tissue interactions may be revealed, along with the likely original defect.

1.5.3 Isolating developmentally important genes

Developmentally important genes may be identified in a number of ways. One popular approach in mammalian genetics has been to isolate genes related to those known to be developmentally important, usually from another species. This homology based approach clearly limits the types of genes that may be isolated. A more general approach is that of obtaining mutants defective in a particular process or structure. This can in itself reveal much about normal development (discussed

above), and the molecular basis for these mutations may be determined using approaches such as positional cloning or functional complementation. Genes may also be identified biochemically from a functional assay that allows purification of a desired activity, and subsequent protein sequencing. Such an approach has been successfully used to isolate the genes for the netrins, a family of chemoattractants, from their neuron outgrowth promoting activity (Serafini *et al.,* 1994; Kennedy *et al.,* 1994) . Expression information can be used to identify genes that may be of interest (reviewed by Kato, 1992). This may take the form of a random screen, such as the screening of enhancer trap lines (Allen *et al.,* 1988; Bellen *et al.,* 1990). Alternatively, desired properties may be incorporated into a screen, such as requiring a difference in expression levels between two tissues (see for example, Smith and Gridley, 1992).

1.6 The Pax genes

1.6.1 A family of developmentally important transcriptional regulators.

Members of the Pax gene family (Walther at al, 1991) have a characteristic paired box near the 5′ end of the gene, encoding a DNA-binding motif, the paired domain (Treisman *et al.,* 1991). The different structures in the Pax gene family are outlined below. Pax genes have predominantly been isolated on the basis of hybridisation at low stringency to a probe from the paired box of the genes at the *Drosophila gooseberry* locus, or using probes from previously isolated Pax genes (reviewed by Walther *et al.,* 1991), but *PAX6* was also isolated as a candidate gene from a positional cloning effort at the aniridia locus (Ton *et al.,* 1991), and biochemical analysis of the transcription factor BSAP showed that it was encoded by the *Pax5* gene (Adams *et al.,* 1992). This latter result provided an important indication that Pax gene products were transcriptional regulators, and is supported by the identification of targets of Pax genes (Zannini *et al.,* 1992; Plaza *et al.,* 1993) and by *in vitro* DNA-binding studies (Dressler and Douglass, 1992; Dozier *et al.*, 1993; Epstein *et al.,* 1994a, 1994b;)

Four of the nine known Pax genes, *Pax1*, *Pax2*, *Pax3*, and *Pax6,* have been found to be deleted or mutated in developmental abnormalities in mouse, rat or human (Balling *et al.,* 1988; Hill *et al.,* 1991; Ton *et al.*, 1991; Epstein *et al.,* 1991; Matsuo *et al.,* 1993; Keller *et al.,* 1994; Sanyanusin *et al.,* 1995). From these and other studies outlined in sections to follow, it is now clear that Pax genes encode a family of developmentally important transcription factors. *Pax1* is essential for the normal development of the axial skeleton (Balling *et al.,* 1991). *Pax2* appears to be

involved in the mesenchyme-to-epithelium transition that occurs during kidney development, as well as being important for retina and optic nerve development (Nornes *et al.,* 1990; Keller *et al.,* 1994; Sanyanusin *et al.,* 1995). *Pax3* has important roles in the development of the neural tube, the production of neural crest, in pigmentation and limb musculature (Epstein *et al.,* 1991). *Pax5* has functions both in B cell development and in the nervous system (Adams *et al.,* 1992; Urbanek *et al.,* 1994). *Pax6* is important for the development of the eyes, nose and olfactory bulbs (Hogan *et al.,* 1986; Hill *et al.,* 1991; Matsuo *et al.,* 1993). *PAX7*, like *PAX3*, is involved in alveolar rhabdosarcoma and may be important in the control of cellular proliferation (Davis *et al.,* 1994). PAX8 controls thyroid specific gene expression (Zannini *et al.,* 1992).

1.6.2 Molecular features of the Pax gene family.

The paired domain was originally identified in *Drosophila* segmentation genes, of the *gooseberry* family and *paired,* and found to encode a DNA-binding motif with a three alpha helix structure (Bopp *et al.*, 1986; Burri *et al.*, 1989; Bopp *et al.*, 1989; Treisman *et al.,* 1991). The vertebrate Pax family encode proteins with a paired domain near the amino terminus, but there are a number of different classes of gene within the family encoding products with distinct combinations of additional motifs, in particular products with or without a conserved octapeptide and with a full *paired*-type homoedomain, partial homeodomain or no homeodomain (Walther *et al.,* 1991). The homeodomain is also a DNA-binding motif (reviewed by Gehring *et al.,* 1990).

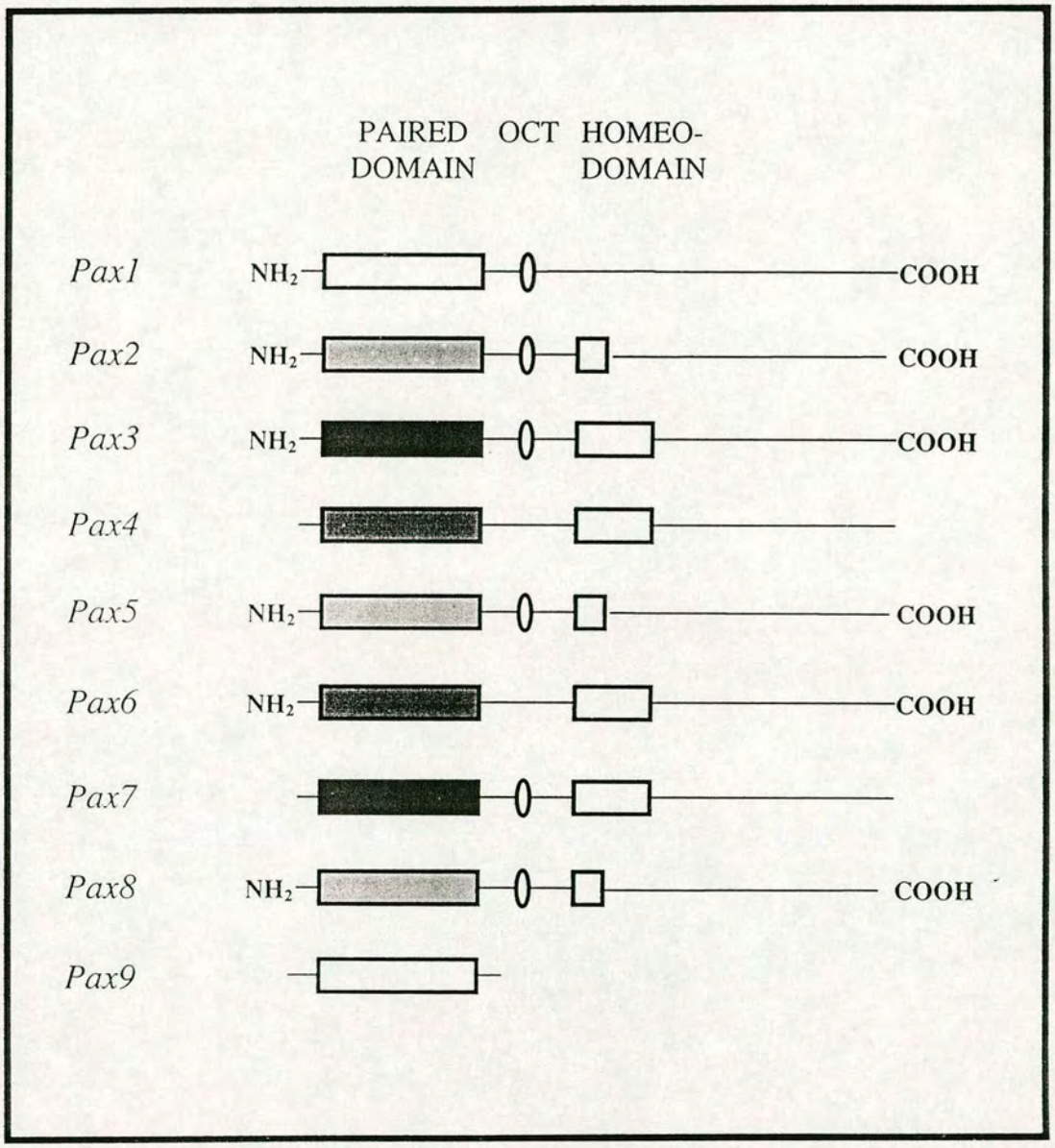

Figure 1.6
Conserved domains in the Pax gene family. Structures of proteins predicted from vertebrate Pax gene DNA sequences.Three principal conserved domains are identified, the paired-domain, the octapeptide motif and the full, or partial, *paired*-like homeodomain. Overall structures and paired-domain sequences are used to group the Pax genes into sub-families, shown by different shading patterns in the paired domain. Adapted from a figure in Stuart *et al.*, (1994)

The structures of major predicted products from the the nine known murine and human Pax genes are summarised in Fig. 1.6 The complete range of motifs is demonstrated by the products of the *Pax3* and *Pax7* genes which encode a paired domain, octapeptide motif and a full *paired*-type homeodomain. *Pax1*,*-2*, *-5* *-8* and *-9* all encode proteins with octapeptide after the paired domain, but form two separate classes, with the products of the *Pax2*, *-5* and *-8* genes having only a partial homeodomain, and that of *Pax1* completely lacking the homeodomain. PAX4 and PAX6 do have a complete homeodomain and paired domain, but lack the octapeptide found on the carboxyl side of the paired domain in the products of all the other Pax genes. Sequence analysis within and outside the paired box suggests that genes within these classes are indeed more related to each other than to those genes with a different overall structure (Gouldling *et al.,* 1991; Walther *et al.,* 1991). PAX-9, whose overall structure has not been determined, is most similar within the paired domain to PAX1 (Stapleton et al, 1993)

It is to be expected from the differences in gene structure, that not all information on the roles of Pax genes will be of equal relevance when considering *Pax6* specifically. The regulated expression of Pax genes during development, and the defects associated with Pax gene mutations, suggest however that the majority of the known Pax genes will have some role during development. In the following sections, information on each of the Pax genes (other than *Pax4* and *PAX9* about which very little is known (Walther *et al.*, 1991; Stapleton *et al*, 1993)), will first be considered separately, with particular emphasis on their possible roles during

development. This will be followed by a description of the *Pax6* gene and the developmental consequences of mutations in it. Subsequent sections will then draw together some of the features common to several Pax genes.

1.6.3 Developmental roles of the *Pax1* gene

1.6.3.1 *Pax1* and the production of axial skeletal elements from scleratome.

Pax1 expression (Deutsch *et al.,* 1988) and the finding that *Pax1* is mutated in *undulated* (Balling *et al.,* 1988), a mouse mutant with axial skeletal defects (Gruneberg, 1954), suggest that *Pax1* is important for the formation of axial skeletal structures from scleratome, before or during the condensation of the scleratome, and prior to chondrogenesis of the vertebral bodies (Gruneberg, 1954; Koseki *et al.,* 1993; Wallin *et al.,* 1994). Further extra-vertebral *Pax1* expression sites and *undulated* phenotypes have been identified (Deutsch *et al.,* 1988; Dietrich *et al.,* 1993; Timmons *et al.,* 1994; Wallin *et al.,* 1994; Dietrich and Gruss, 1995). The phenotypes and expression, together with transformation of cells expressing *Pax1,* suggest that *Pax1* could be important for regulating the proliferation of specific precursor cell populations (Maulbecker and Gruss, 1993; Dietrich and Gruss, 1995). Alternatively, *Pax1* might be important in the early differentiation of affected structures, possibly by controlling condensation of cells (Timmons *et al.,* 1994) .

Presence of a notochord is required for the induction or maintenance of *Pax1* expression, and *Pax1* expression in scleratome is in turn important for notochord development (Koseki *et al.,* 1993; Wallin *et al.,* 1994).

1.6.3.2 Association between *Pax1* expression and *undulated* mutant phenotype.

Pax1 is unusual amongst Pax genes in not being expressed in the developing CNS (Deutsch *et al.,* 1988). Instead, it is expressed mainly in mesodermal tissues, notably during the formation of axial structures, the vertebral bodies and intervertebral discs (Deutsch *et al.,* 1988; Dietrich *et al.,* 1993; Wallin *et al.,* 1994; Dietrich and Gruss, 1995). *Pax1* expression is downregulated as vertebral bodies begin to chondrify, but *Pax1* continues to be expressed in the anlagen of the intervertebral disks and in the perichondrium surrounding the vertebral body, pedicles and proximal ribs (Wallin *et al.,* 1994; Dietrich and Gruss, 1995).

Pax1 is mutated or deleted in three alleles of the mouse mutant *undulated*, *un*, un^{ex} and Un^{s} (Balling *et al.,* 1988, 1992; Dietrich and Gruss, 1995). All three *undulated* alleles have defects in the ventral vertebral column and intervertebral discs. In homozygous *undulated* mice (Un^{s}/Un^{s}, *un/un*, and un^{ex}/un^{ex}), there are shape abnormalities of the vertebral bodies and an absence of intervertebral disks which may lead to vertebral body fusions (Gruneberg ,1954; Wallin *et al.,* 1994; Dietrich and Gruss, 1995). Ribs may be floating or fused directly to the pedicles (Koseki *et al.,* 1993), and in the extreme Un^{s}/Un^{s} mice, absence of the thirteenth pair of ribs occurs.

Extra-vertebral defects found in *undulated* animals also correlate closely with sites of *Pax1* expression. Fusions of the dorsal root ganglia, abnormalities of the pectoral girdle, reductions in the thymus and shortened facial skeleton can be associated with *Pax1* expression found respectively in the connective tissue

surrounding dorsal root ganglia, in the anterior forelimb, in the endoderm of the pharangeal pouches, and in the mandibular and maxillary processes (Deutsch *et al.,* 1988; Dietrich *et al.,* 1993; Timmons *et al.,* 1994; Wallin *et al.,* 1994; Dietrich and Gruss, 1995).

1.6.3.3 Roles for *Pax1* in regulating proliferation or differentiation.

Several *undulated* phenotypes are consistent with *Pax1* regulating proliferation in specific sub-populations of cell. Absences and abnormalities of acromion (a process pointing ventrally from the lateral surface of the scapula), vertebral bodies and intervertebral disks all follow from earlier reductions in blastemal size reductions, and the defects in thymus development are also size reductions (Timmons *et al.,* 1994; Dietrich and Gruss, 1995). Moreover, domains of *Pax1* expression are often sites of high proliferation (Dietrich and Gruss, 1995), and ectopic expression of wild-type *Pax1* is more effective in transforming cells than using the *un* allele (Maulbecker and Gruss, 1993).

In other *undulated* phenotypes, such as the formation of a ligamentous acromion, the balance between forming bone or dense connective tissue is skewed (Timmons *et al.,* 1994). Timmons *et al.,* (1994) have derived a speculative model in which the *Pax1* regulates adhesive properties important for the condensation of mesenchyme, with differential expression of *Pax1* being important maintaining the closely placed chondrogenic domains as distinct elements.

1.6.4 *Pax2*: involvement in kidney and eye development.

Evidence that *Pax2* has roles in kidney and eye development has come from expression studies (Dressler *et al.,* 1990; Nornes *et al.,* 1990; Dressler and Douglass, 1992), inhibition of *Pax2* with antisense oligonucleotides (Rothenpieler and Dressler, 1993), deregulated *Pax2* expression (Dressler *et al.,* 1993), factors found to influence *Pax2* expression (Phelps and Dressler, 1993; Ryan *et al.,* 1995), the phenotype of mice deleted for *Pax2* (Keller *et al.,* 1994) and human developmental abnormalities associated with mutations within *PAX2* (Sanyanusin *et al.,* 1995).

In kidney development, *Pax2* appears to be important for the condensation of induced mesenchymal cells surrounding the ureteric bud, and their subsequent conversion to an epithelium, forming the glomeruli and the proximal and distal tubule. *Pax2* is expressed in the induced mesenchyme, and through this transition, and is downregulated in more mature renal epithelium (Dressler *et al.,* 1990; Dressler and Douglass, 1990). *Pax2* oligonucleotides have an inhibitory effect on both mesenchyme condensation and conversion to epithelium (Rothenpieler and Dressler, 1993). The normal downregulation of *Pax2* expression also appears to be important, as suggested by the continued expression of *Pax2* in epithelial components of Wilms' tumors (Dressler and Douglass, 1992), and the effect on mature nephrons, but not newly formed epithelia, that results from ectopic expression of *Pax2* in transgenic mice (Dressler *et al.,* 1993). The downregulation of *Pax2* transcription may be mediated by the product of the WT1 Wilms' tumor gene (Ryan *et al.,* 1995).

Outside the kidney, *Pax2* is expressed in the CNS and otic vesicle, where strong *Pax2* expression may be related to the patterns of neurogenesis, and in the

optic vesicle, where expression is first present distally before optic cup formation, and then becomes limited to the optic stalk and the ventral half to two-thirds of both inner and outer layers of the optic cup (Nornes *et al.,* 1990). Subsequent *Pax2* expression in the eye is consistent with a role in optic nerve formation. *Pax2* is expressed in the regions that invaginate to form the optic fissure, and then at the exit of optic nerves from the eye, in the optic disk, optic stalk and in cells along the vitreal border of the neuroblastic layer of the central retina (Nornes *et al.,* 1990)

1.6.4.1 Kidney and retinal defects associated with *Pax2* mutations.

Mice with dominant kidney and retinal defects (*Krd*) have been described (Keller *et al.,* 1994), which have a deletion of approximately 7cM in mouse chromosome 19, including deletion of the entire *Pax2* gene. Loss or disruption of other genes might contribute to the phenotype, but a human *PAX2* frame-shift mutation in a family with eye and kidney defects (Sanyanusin *et al.,* 1995) suggests that *Krd* may quite closely approximate the heterozygous null *Pax2* phenotype (Read, 1995).

The phenotype within the family is somewhat variable, but shows many similarities to *Krd* mice, notably hypoplastic kidneys, poor differentiation particularly at the corticomedullary junction, swollen ureters and abnormalities in visually evoked potentials detected by electroretinograms (Keller *et al.,* 1994; Sanyanusin *et al.,* 1995). The phenotype in this family correlates closely with *Pax2* expression, including, for example, a consistent finding of bilateral optic nerve coloboma (Sanyanusin *et al.,* 1995).

1.6.5 Multiple roles for the *Pax3* gene during development.

Pax3 appears to be important for the neural tube development, neural crest migration, and for the development of limb musculature. Evidence for this is a combination of expression pattern (Goulding *et al.,* 1991; Bober *et al.,* 1994; Tsukamato *et al.,* 1994) and the developmental defects resulting from *Pax3* mutations in the mouse mutant *Splotch* (Epstein *et al.,* 1991, 1993; Goulding *et al.,* 1993; Vogan *et al.,* 1993) and human Waardenburg syndrome (Tassabehji *et al.,* 1992; Baldwin *et al.,* 1992; Hoth *et al.,* 1993; reviewed by Farrar *et al.,* 1994). A role in regulation of proliferation is suggested by features of the expression pattern, such as expression limited to mitotic stem cell populations in the dorsal neural tube (Goulding *et al.,* 1991), and by the involvement of *PAX3* in alveolar rhabdosarcoma (Barr *et al.,* 1993; Fredricks *et al.,* 1995).

1.6.5.1 *Pax3* expression and the *Splotch* phenotype.

Pax3 is expressed in dorsal neural tube, in neural crest derivatives such as the dorsal root ganglia, and in the lateral somite from which limb musculature is derived (Goulding *et al.,* 1991). Homozygous *Splotch* animals have neural tube defects, deficiencies in neural crest derivatives, and severely impaired development of limb musculature (Auerbach ,1954; Moase and Trasler, 1989, 1990; Franz, 1989, 1990, 1993; Franz *et al.,* 1993). PAX3 may be important within the dorsal neural tube for neural crest migration (Moase and Trasler, 1990), which would reconcile the apparent absence of *Pax3* expression from the neural crest derivatives of the heart and from migrating melanocytes (Goulding *et al.,* 1991), with persistent truncus

arteriosis and pigmentation defects in *Splotch* animals (Auerbach, 1954; Franz, 1989).

1.6.5.2 Waardenburg syndrome and *PAX3* mutations.

Waardenburg syndrome (WS) is a dominantly inherited syndrome of deafness, pigmentary abnormalities, such as a white forelock or heterochromic irises, and characteristic facial features (reviewed by Pantke and Cohen, 1971). WS is clinically variable and divided into subtypes, two of which, WS type I and WS type III, can result from mutations in PAX3 (see Farrar *et al.,* 1994 for review). WS type I patients have dystopia canthorum, in which the inner corners of the eye displaced outwards and the bridge of nose is unusually broad. About two third of these patients have pigmentation abnormalities and similar proportion have hearing defects. WS type III, or Klien-Waardenburg syndrome, patients have WS type I with additional limb abnormalities. Rarely, WS type I patients have neural tube defects similar to those found in *Splotch* homozygous mutant mice (Pantke and Cohen, 1971; Narod *et al.,* 1988; Begleiter and Harris, 1992; Moline and Sandlin, 1993). None of these neural tube defect patients have yet been analysed for *PAX3* mutations, but from the many different *PAX3* mutations found in Waardenburg syndrome, there is so far little correlation between the nature of the mutation and the severity of phenotype (Farrar *et al.,* 1994; Read *et al.,* 1995). Many of the mutations do however result in amino acid substitutions, rather than major disruptions of the gene product, perhaps suggesting that small reductions in effective dosage may be sufficient to produce a phenotype (Read, 1995).

1.6.5.3 *Pax3* involvement in Alveolar rhabdosarcoma.

Pax3 is also involved in alveolar rhabdosarcoma, a tumour that characteristically includes regions of striated muscle differentiation. Consistent translocations to the last *PAX3* intron (between exons 7 and 8) juxtapose *PAX3* with sequences encoding the carboxyl terminal region of the transcription factor FKHR (Barr *et al.,* 1993). A hybrid message is produced which is expected to yield a chimeric protein product retaining the DNA-binding motifs of PAX3 (Barr *et al.,* 1993). Such a chimeric protein acts as a more potent transcriptional activator than wild-type PAX3 (Fredricks *et al.,* 1995). Both the constituents of the tumor, and its molecular basis, could be consistent with the chimeric protein upregulating some of the normal targets of PAX3, suggesting a role for the *Pax3* gene in controlling cellular proliferation (Barr *et al.,* 1993; Fredricks *et al.,* 1995).

1.6.6 *Pax5* is important for B-cell development and in the midbrain-hindbrain region.

Two roles have been identified for the *Pax5* gene, in B cell development and in the development of the midbrain-hindbrain region. Adams *et al.,* (1992) found that *Pax5* encoded the B-cell lineage specific transcription factor, BSAP, that is expressed in early B cell differentiation (Barberis *et al.,* 1990), and regulates B cell proliferation (Wakatsuki *et al.,* 1994). In the developing nervous system *Pax5* expression was found to be expressed at the midbrain -hindbrain boundary, where the related genes *Pax2* and *Pax8* were not expressed (Adams *et al.,* 1990; Asano and Gruss, 1992; Nornes *et al.,* 1990; Plachov *et al.,* 1990). Urbanek *et al.,* (1994) created PAX5 deficient mice in which early B cell differentiation was completely blocked, and patterning of the posterior midbrain was disrupted.

The place of the *Pax5* gene in the lymphopoiesis has been reviewed by Dorshkind, (1994). Both B and T lympocytes are descendants of pluripotental hematopoietic stem cells that also produce the myeloid lineages. The B cell differentiation pathway itself can be divided into a number of stages (Pro-B cell; Early pre-B cell; Late pre B cell; B cell) on the basis of characteristic expression of cell surface markers. In PAX5 deficient mice, B cell differentiation however is blocked before the transition to early pre-B cells, but since there are no defects in T cell differentiation, the requirement for *Pax5* can be placed after the hypothesised bipotential-precursor stage (Urbanek *et al.,* 1994). Overexpression of *Pax5* and culturing with antisense oligonucleotides against the *Pax5* translation initiation start

site have positive and negative effects respectively on B cell proliferation, suggesting that *Pax5* is a regulator of B cell proliferation (Wakatsuki *et al.,* 1994).

One target of BSAP/PAX5 regulation is the *CD19* gene (Kozmik *et al.,* 1992). CD19 normally forms part of a multimeric signal transduction complex (Kozmik *et al.,* 1992) and is not expressed in cells of the *Pax5*-mutant lymphocyte lineage (Urbanek *et al.,* 1994). The rearrangement and expression of immunoglobulin genes is also important in B cell development, and potential BSAP/PAX5 binding sites are found in several regulatory regions of immunoglobulin heavy chain locus (Liao *et al.,* 1992; Rothman *et al.,* 1991; Singh and Birshtein, 1993; Waters *et al.,* 1989; Xu *et al.,* 1992a). PAX5 binding to these sites may act as a repressor of immunoglobulin gene expression during early B cell development. (Singh *et al.,* 1993). Binding to the switch regions of the immunoglobulin heavy chain genes could even indicate a role in the regulation of B cell-specific recombination in immunoglobulin class switching events (Xu *et al.,* 1992a).

Pax5 is expressed at the midbrain-hindbrain boundary (Asano and Gruss, 1992), in a similar pattern to that observed for the closely related zebrafish gene *pax[zf-b]* (Krauss *et al.,* 1991 a, c; Asano and Gruss, 1992). Since they found reduction of the posterior midbrain in homozygous *Pax5*-mutant animals but did not find any evidence of increased cell death or an imbalance in cell types, Urbanek *et al.,* (1994) suggest that *Pax5* is important for proliferation in this region.

1.6.7 *Pax7*: Similarity to *Pax3* and involvement in Alveolar rhabdosarcoma.

Pax7 is closely related to *Pax3* both in overall structure and at the amino acid sequence level (Jostes *et al.,* 1991; Goulding *et al.,* 1991). Similarity between these genes extends to the expression patterns, where both genes are expressed both in dorsal nervous system and in dermomyotome (Jostes *et al.,* 1991; Goulding *et al.,* 1991; Bober *et al.,* 1994). The expression patterns are achieved by a common mechanism. Expression of both *Pax3* and *Pax7* in dermomyotome can be induced by contact with surface ectoderm and a diffusible signal from the dorsal neural tube (Fan *et al.,* 1994). Like *PAX3*, *PAX7* is also implicated in alveolar rhabdosarcoma, again by a translocation with the *FKHR* gene (Davis *et al.,* 1994). Some roles of *Pax7* may be similar to those of *Pax3*, but there are also significant differences, for example *Pax7* is not expressed in the dorsal-most neural tube, and consequently does not appear to be expressed in neural crest derived structures (Jostes *et al.,* 1991).

1.6.8 *Pax8* in kidney morphogenesis and the control of thyroid specific genes.

The results of mutations in the *Pax8* gene have not been described. Mapping studies in mouse (Plachov *et al.,* 1990; Walther *et al.,* 1991) placed it near the *Danforth's short tail* (*Sd*) locus, but *Pax8* has since been excluded as a candidate gene for this locus (Jane Alfred, personal communication). Information about the role of *Pax8* comes instead from studies of its expression, and the identification of two probable targets. There is considerable similarity between *Pax2* and *Pax8* expression in the developing kidney and in Wilms' tumours, but the dynamics of the expression suggest that *Pax8* might act slightly later than *Pax2* (Plachov *et al.,* 1990; Poleev *et al.,* 1992). *Pax8* is also expressed in the thyroid (Plachov *et al.,* 1990; Zannini *et al.,* 1992), where it appear be involved in the regulation of two genes expressed there, *thyroglobulin* and *thyroperoxidase* (Zannini *et al.,* 1992). Unlike *Pax2*, no expression of *Pax8* has been reported in the developing eye (Plachov *et al.,* 1990).

1.6.9 The *Pax6* gene

1.6.9.1 Structure of the *Pax6* gene

The human and mouse *Pax6* genes were isolated independently at about the same time, the former as a candidate gene from the human aniridia locus (Ton *et al.,* 1991) and the latter on the basis of homology to *Drosophila gooseberry* genes (Walther and Gruss, 1991). *Pax6* produces a message of about 3kb which, like *gooseberry*, encodes a protein 422 amino acids long with two DNA-binding motifs, a 128 amino-acid paired domain and a 61 amino-acid paired-like homeodomain (Walther and Gruss, 1991; Ton *et al.,* 1991; Glaser *et al.,* 1992).

The mouse and human products are highly similar, (discussed below) and their structural features will be considered together. The paired domain is very close to N terminus of the protein, starting only three amino-acids from the initial methionine. Significantly, at the start of the paired box is at an intron-exon boundary, perhaps suggesting that this motif has at one time been a movable cassette (Glaser *et al.,* 1992; Epstein *et al.*, 1994). Other Pax genes have a conserved octapeptide motif following the paired domain (Walther *et al.,* 1991), but PAX6 lacks this feature. Instead there is 78 amino-acid, glycine and glutamine-rich, linker separating the paired domain from the paired-like homeodomain. The main feature at the carboxyl terminus is a region rich in proline, serine and threonine residues (Hill *et al.,* 1991) which appears to act in transcriptional activation by PAX6 (Glaser *et al.,* 1994)

The *Pax6* transcription unit is complex with 14 exons (Glaser *et al.,* 1992). One major alternative message is produced, inserting exon 5a into the paired box,

which adds 14 amino acids to the paired domain (Walther and Gruss, 1991). Ocular defects in a family with exon 5a splice acceptor site mutations suggest that the ratio of these two products is important (Epstein *et al.,* 1994). The archetypal paired domain contains at least three alpha-helical regions (Bopp *et al.,* 1989; Burri *et al.*, 1989). Epstein *et al.,* (1994) have studied the DNA-binding properties of the two isoforms of the PAX6 paired domain, and found that whereas a paired domain without the 14aa peptide binds to DNA mostly through the amino terminus, the paired domains that contains the exon 5a peptide binds to a distinct consensus sequence via its carboxyl terminus. Thus the exon 5a product appear to act as a molecular toggle altering the DNA-binding specificity of PAX6 (Epstein *et al.,* 1994).

1.6.9.2 High degree of evolutionary conservation of PAX6

Predicted protein products of *Pax6* genes from different vertebrate species show extremely high levels of identity, and most amino-acid substitutions that have occurred between them are conservative ones. Mouse and rat PAX6 amino-acid sequences are identical (Matsuo *et al.*, 1993). Human and rodent PAX6 proteins differ only by a single amino acid, and this is within the peptide encoded by the alternative exon 5a (Glaser *et al.,* 1992). Between human and zebrafish, which diverged more than 400 million years ago, there are rather more differences, but the proteins are still 96% identical, or 97.2% similar if conservative amino acid changes are included (Krauss *et al.,* 1991a; Glaser *et al.,* 1992). The homeodomains of these proteins are identical and there is only a single amino acid substitution in the paired domain (Glaser *et al.,* 1992).

Interestingly, homeodomain sequences show that *Pax6* is rather more closely related to *gooseberry* than to *paired*, but is still less closely related to these two *Drosophila* genes than either vertebrate *Pax3* or *Pax7* are (Krauss *et al.,* 1991b). *Pax6* and these other Pax genes have probably evolved from distinct genes in the common ancestor of vertebrates and *Drosophila*. In addition to the *paired*, *gooseberry*, *pox meso* and *pox neuro* genes related to Pax genes, the fruit fly has a separate *Pax6* homologue, isolated by Quiring *et al.,* (1994) and found to be disrupted in the *eyeless* mutation. *Drosophila* PAX6 protein has a paired domain and homeodomain that are respectively 94% and 90% identical to the mouse PAX6 domains (Quiring *et al.,* 1994). This sequence conservation extends to a functional level, as ectopic expression of either mouse or *Drosophila Pax6* in *Drosophila* imaginal discs causes ectopic eye formation (Halder *et al.,* 1995).

The high degree of conservation of *Pax6* argues that not only is PAX6 vital for the organism, but almost any mutation in *Pax6* results in sufficient disadvantage to the organism for that mutation to be selected against, especially if the mutation alters the DNA binding motifs. Important roles for vertebrate *Pax6* genes in eye development, nasal development and brain development are suggested by the *Pax6* expression patterns and the phenotypes that result from *Pax6* mutations. As discussed below however, these mutations are generally major disruptions of the PAX6 product and so the phenotypic consequences of single amino-acid substitutions are largely unknown.

1.6.9.3 Expression of vertebrate *Pax6* genes.

Pax6 mRNA expression is developmentally regulated as has been reported from work using a variety of vertebrate species including humans (Ton et al., 1991), mouse (Walther and Gruss, 1991, Stroyeva and Gruss, 1994), rat (Matsuo *et al.,* 1993), zebrafish (Krauss *et al.,* 1991a, b; Puschel *et al.,* 1992), chick (Li *et al.*, 1994), quail (Martin *et al.,* 1992) and urodeles (Del Rio-Tsonis *et al.,* 1995). The results from these studies are remarkably similar when equivalent developmental stages are considered, suggesting that the regulation of *Pax6* expression may be strongly conserved, just as the sequence of the protein product is (Puschel *et al.,* 1992).

When the work in this thesis was initiated, only a preliminary study of *Pax6* expression was available, which showed expression in the lens, cornea and neural retina of human embryos (Ton *et al.,* 1991). A much more detailed account was then published for mouse embryos by Walther and Gruss, (1991) who established the time of first expression of *Pax6*, at about embryonic day eight (E8) in the mouse, described expression in the developing nervous system and eye as chronological series, and identified *Pax6* expression at other sites such as the developing olfactory epithelium and pancreas. *Pax6* is thus not expressed in mesoderm but is expressed in ectoderm and endoderm derivatives.

Pax6 expression in the eye implicates the gene in important events in the development of the eye. In the lens *Pax6* is expressed in the developing lens placode and lens vesicle, but is downregulated as lens fibres are produced, being retained in the lens precursor cells of the lens epithelium (Walther and Gruss, 1991). Wholemount *in situ* hybridisation in the chick (Li *et al.,* 1994) suggests that *Pax6*

may define the lens forming region, but also suggests that this surface ectoderm expression is independent of the influence of the optic vesicle. Strong *Pax6* expression within the developing neural retina suggests that *Pax6* is also important in this structure (Ton *et al.,* 1991; Walther and Gruss, 1991; Martin *et al.,* 1992)

Walther and Gruss, (1991) showed that *Pax6* expression defined domains in the developing CNS. They describe how *Pax6* expression in the developing spinal cord becomes limited to domains ventral of the sulcus limitans from an earlier pattern that excluded only the presumptive floor plate and dorsal-most neural tube. These dorso-ventral patterns have attracted particular interest, since they may reveal the mechanisms by which dorso-ventral patterning is established in the spinal cord. *Pax6* expression has been found to positively regulated by signals produced by the notochord, perhaps via the floor plate (Goulding *et al.*, 1993).

Variation of *Pax6* expression along the antero-posterior axis of the CNS was also highlighted by Walther and Gruss(1991), particularly the strong *Pax6* expression in the developing ventral thalamus. This antero-posterior variation is a prominent feature of expression in the zebrafish embryo (Krauss *et al.,* 1991a,b) and is more readily apparent in this species since there are fewer flexures of the brain.

1.6.9.4 Vertebrate *Pax6* mutations are inherited as semidominants.

Mutations within, or deletion of, the *Pax6* gene have been identified in mouse and rat *Small eye* (Hill *et al.*, 1991; Matsuo *et al.*, 1993) and in a variety of human abnormalities of the eye including aniridia (congenital lack of iris) and Peter's anomaly (central corneal opacities)(Ton *et al.*, 1991; Jordan *et al.*, 1992; Glaser *et al.*, 1992, 1994; Hanson *et al.*, 1993, 1994a; Davis and Cowell, 1994). In each of

these species the mutations behave as semidominants (Hogan *et al.*, 1986; Matsuo *et al.*, 1993; Glaser *et al.,* 1994). Not all of the phenotypes have been equally well characterised, but in all three species the heterozygous mutant phenotype includes eye defects, and homozygosity for *Pax6* mutations results in absence of eyes, nasal defects and absence of olfactory bulbs (Hodgson and Saunders, 1980; Hogan *et al.*, 1986; Hill *et al.*, 1991; Schmahl *et al.,* 1993; Matsuo *et al.*, 1993; Fujiwara *et al.*, 1994; Glaser *et al.,* 1994).

1.6.9.5 Aniridia phenotype and mutations

Aniridia is characterised by hypoplasia of the iris (for a clinical review see Nelson *et al.*, 1984). It is a rare disease, affecting 1:60,000 to 1:100,000 of the population. About two thirds of cases are familial, the remainder are sporadic cases without family history. Iris abnormalities may be present at birth but the aniridia phenotype is both progressive and panocular. Lens, cornea, filtration apparatus, and retina can all be affected. Cataracts, corneal opacifications and glaucoma may all occur in aniridia patients, and these problems worsen with age. There is reduced visual acuity and early onset nystagmus resulting from hypoplasia of the fovea and optic nerve.

Many sporadic cases carry cytogenetically visible deletions of chromosome 11 around band p13. Most of these patients have the contiguous gene syndrome WAGR, with predisposition Wilms' tumour (a childhood kidney tumour), aniridia, geniotourinary abnormalities, and mental retardation. Positional cloning, making use DNA from patients with different chromosomal abnormalities and different combinations of the WAGR phenotypes, allowed candidate genes for both Wilms'

tumour susceptibility, (*WT1*) and aniridia, (*PAX6*) to be identified (Call *et al.,* 1990; Gessler *et al.,* 1990; Ton *et al.,* 1991, see Hanson *et al,* (1994b) for a review of the effort to identify the aniridia gene).

Many deletions and intragenic mutations of *PAX6* have now been identified in both sporadic and familial aniridia cases (Jordan *et al.,* 1992; Glaser *et al.,* 1992; Hanson *et al.,* 1993). The spectrum of intragenic mutations has been recently reviewed (Glaser *et al.,* 1995; Hanson and van Heyningen, 1995). There are few missense mutations. Instead stop-codons, frameshifts and splice errors predominate, giving predicted products that are truncated at the carboxyl terminal end. These mutations are scattered throughout the length of the *PAX6* gene however, so truncations occur at many different points. There is little correlation between phenotype and position of the mutation so the majority of these truncations appear to behave equivalently.

1.6.9.6 Peters' Anomaly and defects of the anterior segment of the eye

Three reports (Hanson *et al.,* 1994a; Epstein *et al.,* 1994; Glaser *et al.,* 1994) have implicated *PAX6* in abnormalities of the anterior segment of the eye that do not involve complete absence of iris.

Hanson *et al.,* (1994a) describe a patient with Peters' anomaly (central corneal opacities) who carries a deletion of the *PAX6* gene, and also described a family in which a range of anterior segment defects of the eye, including Peters' anomaly, segregate with a single base change in *PAX6*. The other phenotypes include iris hypoplasia, glaucoma and micropthalmia and a prominent line of fusion of the choroidal fissure, in various combinations. The base change causes a non-

conservative replacement of a arginine by glycine at the N terminal end of an predicted alpha helix structure important for paired domain DNA-binding *in vitro* (Treisman *et al.,* 1991; Hanson *et al.,* 1994a). Glaser *et al.,* (1995) considers this to be a hypomorphic mutation, but the finding of Peter's anomaly with both this mutation and a deletion suggests that it is close to being a complete loss of function (Hanson *et al.,* 1994a).

Glaser *et al.,* (1994) found a mild phenotype of late onset cataracts associated with a partial loss of the PAX6 transactivation domain activity. An alteration in the ratio of splice forms with and without exon 5a, discussed above, also leads to a mild phenotype, compared with aniridia (Epstein *et al.,* 1994).

1.6.9.7 Phenotype of mouse *Small eye*.

As the name suggests, mice heterozygous for the *Small eye* mutations have micropthalmia, but there are also anterior polar cataracts (Jack Favor, personal communication; Hogan *et al.,* 1986). Small vacuolated lenses are formed, and there may be adhesions between lens and cornea, and abnormal folding of the retina (Hogan *et al.,* 1986; Hogan *et al,* 1988). In the homozygous mutant embryos, eyes, nasal cavities and olfactory bulbs are all absent (Hogan *et al.,* 1986; Schmahl *et al.,* 1993). The absence of lenses and nasal cavities has been suggested by Hogan *et al.,* (1986) to result from a failure to form lens and nasal placodes. Clayton and Campbell (1968) suggested that there might be a defect in extracellular membrane synthesis in *Small eye* mice, but analysing laminin expression, Hogan *et al.,* (1986) found no such defect.

An analysis of brain development in *Small eye* mice found defects in the development of the cerebral cortex in both heterozygous and homozygous animals (Schmahl *et al.,* 1993). At late gestation the germinative epithelium of the cerebral hemispheres was enlarged, apparently reflecting a defect in the migration of some post-mitotic cells to form the cortical plate (Schmahl *et al.,* 1993). In heterozygous animals the defect may be a delay, as the abnormalities are not apparent shortly after birth. In homozygous animals the defect is more severe and mirrors a similar defect in the germinative epithelium of the cerebellum (Schmahl *et al.,* 1993).

1.6.9.8 *Small eye* mutations.

The first conclusive evidence of *Pax6* involvement in eye development came from the analysis of the *Small eye* mutation by Hill *et al.,* (1991). They found that not only was the *Pax6* gene deleted in a radiation induced allele, Sey^{H}, but there were intragenic mutations in two further alleles, both predicted to cause premature termination of the translated product. In the original *Sey* allele there is a single G→T transversion producing a stop codon upstream of the homeobox (Hill *et al.,* 1991). Sey^{Neu}, an ENU induced allele also has a G→T change, but in a splice donor site towards the 3´ end of the gene, preventing the intron containing the mutation from being spliced. As a result the predicted protein product has a novel 22 amino acid, intron-encoded peptide replacing the proline, serine and threonine rich transactivation domain encoded by exons 11, 12 and 13 (Hill *et al.,* 1991; Glaser *et al.,* 1992; Glaser *et al.,* 1994).

1.6.9.9 Rat *Small eye*

The rat *Small eye* allele described by Matsuo *et al.,* 1993 is a single base insertion downstream of the homeobox which has similar consequences to the mouse Sey^{Neu} allele. The insertion creates a novel splice donor leading to internal deletion of the *Pax6* coding region and thus loss of the proline, serine and threonine-rich transactivation domain. Rat *Small eye* phenotype is similar to the mouse but two important findings come from this species. In homozygous animals there is a failure of midbrain neural crest cells to migrate into the nasal region (Matsuo *et al.,* 1993). Furthermore, surface ectoderm isolated from the eye region of homozygous mutants is unable to form a lens, even in the presence of wild-type optic vesicles (Fujiwara *et al.,* 1994)

1.6.9.10 Phenotypic similarities of *Pax6* mutations in different species

There are considerable phenotypic similarities between the *Pax6* mutations in different species, but there is also some variation between species, between individuals with the same mutation and even between left and right eye of the same individual. The phenotype of *Small eye* mice can include many of the features found with human *PAX6* mutations, the cataracts and iris hypoplasia typical of aniridia patients (Jordan et al., 1992) and the central corneal opacities and adhesions between cornea and lens found in Peters' anomaly (Hanson *et al.*, 1994a). Micropthalmia is a consistent feature of both the mouse and rat *Small eye* (Roberts *et al.,* 1967; Clayton and Campbell, 1968; Matsuo *et al.,* 1993) but has not been associated with mutation or loss of human *PAX6*, except in one patient described by Hanson *et al.,* (1994a).

The variation in phenotype is shown by the observation that deletion of the *PAX6* gene can result either in Peters' anomaly (Hanson *et al.,* 1994a) or in aniridia (Ton *et al.*, 1991). Equally the same *Small eye* allele can result in either or both phenotypes (Jordan *et al.,* 1992; Hanson *et al.,* 1994a). Within single pedigrees of familial aniridia the phenotype can vary from merely a thinning of the iris, through irregular hypoplastic irises, to complete absence of the iris, all either with or without other ocular defects (Hitter *et al.,* 1980).

1.6.9.11 Haploinsufficiency for *PAX6*

The molecular basis of the known mutations and comparison of their phenotypic consequences both point to semidominance being due to haploinsufficiency for *PAX6*. The phenotypic similarity of point mutations and *Pax6* deletions in *Small eye* (Hill *et al.,* 1991) aniridia (Hanson *et al.*, 1992) and Peters' anomaly (Hanson *et al.*, 1994a) suggest that the point mutations are probably loss of function alleles.

The extremely high conservation of the PAX6 protein sequence implies that alteration of almost any residue will have deleterious consequences for the organism. A number of authors have noted however that the majority of the intragenic *Pax6* mutations described are predicted to result either in the deletion of important domains or in premature termination of the translated product (Glaser *et al.*, 1995; Read 1995; Hanson and van Heyningen, 1995). From twenty nine mutations reviewed by Glaser *et al.,* (1995) only two are missense mutations (Hanson *et al.,* 1993; Hanson *et al.,* 1994a). This bias, together with the conservation of PAX6,

suggest that the phenotypes so far examined for *Pax6* mutations are not representative of the possible *Pax6* mutant phenotypes.

Truncated products might, in principle, act in a dominant-negative fashion, for example by sequestering co-factors required for normal activity or binding with wild-type PAX6 protein to produce inactive complexes. The range of different truncation and deletion products all having the same phenotype argues against this however, and thus suggests that the *Small eye* and aniridia mutations are mainly null or severely hypomorphic alleles.

Aniridia may also occasionally result from a position effect following a chromosomal rearrangement (Sarah Danes, personal communication). If the *Pax6* gene was found to be silenced by such chromosomal events, this would also be consistent with a haploinsufficiency.

1.6.10 Pax genes are developmentally regulated.

The Pax genes are a family of genes (Walther *et al.,* 1991) encoding transcription factors, that are temporally and spatially regulated during development. Most are expressed along a large portion of the antero-posterior axis (Nornes *et al.,* 1990; Goulding *et al.,* 1991; Adams *et al.,* 1990; Walter and Gruss, 1991; Jostes *et al.,* 1991; Plachov *et al.,* 1990). Some are important for processes that occur along this axis, such as *Pax1* in vertebral body formation and *Pax3* in neural crest migration. Unlike the Hox genes however, which have major roles in axial patterning (see McGinnis and Krumlauf, 1992 for a review), the expression pattern and role of many Pax genes are better described as organ- or tissue-specific. The *Pax1* and *Pax3*

roles described above can be seen in this way, as can *Pax2* and *Pax8* in kidney development, *Pax5* in the B-cell lineage and *Pax2* and *Pax6* in eye development.

1.6.11 Little redundancy in Pax gene expression.

There is reason to expect some redundancy between the Pax genes as they may recognise similar targets (Epstein *et al.,* 1994) and can be expressed in overlapping domains. This is especially true within each class of Pax genes, where there is often both greater structural similarity and an overlap of expression. Examples include the *Pax2* and *Pax8* expressed in the developing kidney and *Pax3* and *Pax7* in dermomyotome and dorsal neural tube.

Pax9 expression has not been reported, but so far, *Pax1* shows little overlap in expression with other Pax genes. The *undulated* phenotype can thus be expected to quite accurately reflect the requirement for *Pax1* during development. No abnormality was found in pelvis but there are defects in the jaw, thymus, vertebral column and pectoral girdle, thereby covering the whole range of sites of *Pax1* expression (Timmons *et al*, 1994; Dietrich and Gruss, 1995). This suggests that there is very little redundancy within the pattern of *Pax1* expression, i.e. that *Pax1* is important almost everywhere it is expressed.

Similarly *Pax3* mutations give defects in neural tube, neural crest and limb musculature (Epstein *et al.*, 1991), *PAX2* mutations affect both eye and kidney (Keller *et al.*, 1994; Sanyanusin *et a.l*, 1995), and *Pax5* is required both in the B-cell lineage and at the midbrain-hindbrain boundary (Urbanek *et al.*, 1994). Together these results suggest that Pax gene expression patterns may predict the requirement for the genes quite accurately.

1.6.12 Pax gene expression in proliferating and immature cell types.

Most expression studies are confined to embryonic development where there are few terminally-differentiated cells, particularly in the brain (Ponte *et al.,* 1994). Nevertheless expression studies do suggest that Pax genes are rarely expressed in terminally differentiated cells, but rather are expressed in stem cell population, recently post-mitotic cells or immature cell types. 1) *Pax1* expression in scleratome and anterior limb mesenchyme is downregulated with the onset of chondrogenesis (Deutsch *et al.,* 1988; Timmons *et al.,* 1994). 2) In the developing kidney *Pax2* is not expressed in mature kidney tubule epithelium, but is expressed in the stages producing that epithelium from the induction of mesenchyme. *Pax8* expression is present somewhat later but is similarly restricted to intermediate stages of the same process (Poleev *et al.,* 1992). 3) *Pax3* is expressed in myoblast stem cells destined to populate the developing limbs (Bober *et al.,* 1994). 4) In the nervous system *Pax2* expression correlates with the transition from predominantly mitotic to migratory behaviour (Nornes *et al.,* 1990). *Pax3* and *Pax7* CNS expression is limited to mitotic stem cells of the ventricular zone and are rapidly downregulated prior to neuroblast migration (Goulding *et al.,* 1991; Jostes *et al.,* 1991). Similarly, the defects in neuronal migration in the *Small eye* cerebral cortex have been suggested to occur as cells undergo the transition from a postmitotic to a migratory phenotype (Schmahl *et al.,* 1993). 5) In lymphopoesis, *Pax5* is not expressed either in the earliest pluripotent haematopoetic precursors of B and T cells, nor in mature plasma cells, but rather is expressed in the immature cell types in between (Barberis *et al.,* 1990). There are examples of Pax gene expression in adults, for example *Pax8* expression in the

thyroid (Zannini *et al.*, 1992). Generally however expression studies suggest that Pax genes are not required in mature cell types, but are active in their precursors.

1.6.13 Pax genes and the control of proliferation.

Pax gene expression in proliferating precursor populations, as well as aspects of the Pax mutant phenotypes and the involvement of Pax genes in oncogenesis, all suggest that Pax genes may normally regulate cell proliferation.

The common feature of several *undulated* phenotypes is a reduction in the size of the affected structures (Dietrich and Gruss, 1995). An elongation of cell cycle time has also been shown in the neural tube of *Splotch* mutants (Wilson, 1974). The involvement of Pax genes in oncogenesis has been reviewed by Stuart *et al.,* (1994). Cells can be transformed by overexpression of *Pax* genes (Maulbecker and Gruss, 1993), and also by production of chimeric *PAX3* and *PAX7* proteins with stronger than normal transactivation activities (Barr *et al.,* 1993; Davis *et al.,* 1994; Fredricks *et al.,* 1995) . The Pax genes seem to be particularly sensitive to effective gene dosage, since all mutations so far described are inherited as semidominants. The experimental demonstrations of oncogenic potential and alveolar rhabdosarcomas may both represent increases in effective gene dosage, perhaps by stronger than normal activation of normal targets. This suggests that Pax genes could be normally involved in regulation of proliferation.

1.6.14 Pax gene regulation of the extracellular environment.

In several cases it appears possible that Pax genes govern adhesive properties or the expression of cell surface molecules. *Pax1* and *Pax2* mutant phenotypes

involve failures of condensation (Gruneberg, 1954; Rothenpieler and Dressler, 1993; Timmons *et al.,* 1994). Cell signaling could be at fault, but alterations in laminin expression upon *Pax2* overexpression (Dressler *et al.,* 1993) may indicate that such genes are targets of PAX2. Similarly there are cell migration defects in *Pax3* mutants, both in neural crest and skeletal muscle precursors. These could be due to abnormal adhesion, and NCAM isoform expression is indeed altered in *Splotch* mutants. (Moase and Trasler, 1991)

Extracellular matrix, basement membrane and cell surface molecules are well represented amongst cloned genes and immunological reagents. Thus changes in these properties in mutants may either accurately reflect the nature of Pax gene targets, or be simply a function of the tools available to researchers. For *Pax5* however, immunoglobulin genes and the cell surface molecule CD-19 have been defined as targets by binding studies (Liao *et al.,* 1992; Rothman *et al.,* 1991; Singh and Birshtein, 1993; Waters *et al.,* 1989; Xu *et al.,* 1992a; Kozmik *et al.,* 1992). Thus in at least some cases Pax gene expression is reflected at the cell surface.

1.6.15 Defined Pax gene targets are tissue specific.

There are few known targets of vertebrate Pax genes and consequently it is difficult to identify general properties. It is easier to exclude genes as being general targets. In particular, the identified targets such as those of *Pax5* in the B-cell lineage (Kozmik *et al.,* 1992)and *Pax8* in the thyroid (Zannini *et al.,* 1992), are tissue-specific and are unlikely to be regulated by other Pax genes not expressed in those tissues. Some targets of invertebrate genes can also be excluded as candidates for regulation by vertebrate Pax genes. *TSAP* targets are developmentally regulated

histone genes, which have not been found in any other higher eukaryotes, and so are unlikely to have equivalents in vertebrates (Barberis *et al.,* 1989,1990).

1.6.16 Regulation of Pax genes by notochord or floor plate signals

Four Pax genes, in different systems, appear to respond to signals from the notochord or floor plate. In scleratome, *Pax1* expression depends upon the notochord either for its activation or its maintenance (Koseki *et al.,* 1993; Brand-Saberi *et al.,* 1993). Ectopic ventral basal plate domains of *Pax2* expression in regions of *Danforth's short tail* mice lacking a floor plate suggests regulation by notochord or floor plate (Phelps and Dressler, 1993). Also in the developing spinal cord, *Pax3* and *Pax6* expression domains are both altered in response to notochord grafts (Goulding *et al.,* 1993). The notochord and floor plate express *Sonic hedgehog* (*Shh*). There is evidence from ectopic expression studies that Shh can induce floor plate expressed genes (Echelard *et al.,* 1993; Roelink *et al.*, 1994), enhance the formation of scleratome, and antagonise the expression of dermotome. These latter effects of *Shh* are judged in part by the intensification of *Pax1* expression and repression of *Pax3* expression in dermomyotome (Johnson *et al.,* 1994) or in cultured presomitic mesoderm (Fan *et al.,* 1994). Other signals might also affect Pax gene expression. Dorsal neural tube and surface ectoderm can induce *Pax3* and *Pax7* dermomyotome expression (Fan *et al* , 1994). The response of *Pax2*, *Pax3* and *Pax6* to BDNF and NGF in primary cultures of mouse cerebellum suggests that these factors could also be involved in Pax gene regulation (Kioussi and Gruss, 1994). As well as being a source of Shh, the floor plate is also a site of retinoid synthesis (Wagner *et al.,* 1990) which equally might influence patterning in the surrounding region.

1.6.17 Common aspects of the processes involving *Pax* genes.

The Pax genes are important for the development of a wide range of structures and cell types. Superficially, there appears to be little to link these various roles, even two roles of the same gene, such as *Pax5* in B cell development and brain development (Urbanek *et al.,* 1994). Nevertheless the sections above demonstrate that many similarities may exist between genes within the Pax family, which might lead to common aspects of their diverse roles being identified. For example, with no less than five Pax genes (of the seven well characterised ones) responding to signals produced by notochord and floor plate or by dorsal ectoderm, it is possible that Pax genes could be mediating the response of cells to such signals. Similarly, since gene dosage is critical for most Pax genes, major changes in cell behaviour might be brought about by relatively minor changes in Pax gene expression. Pax genes might thus be important at major transitions in cell behaviour, such as from mitotic to migratory phenotypes or in the formation of epithelium from mesenchyme. At present, these generic models of Pax gene action must remain speculative. A clearer indication of the roles of specific Pax genes is required, from which it should be possible to identify what is in common with other Pax genes, and what is unique to that gene.

1.7 Aims of this work

When this project was initiated it was apparent from the identification of *Pax6* mutations in *Small eye* mutants (Hill *et al.,* 1991) that *Pax6* was in some way important for the development of the eye, nose and brain. Very little was known

about the *Pax6* gene however. It was known that human *PAX6* was expressed in the developing eye (Ton *et al.,* 1991), but there was no indication of what the relationship of *Pax6* to defects in nasal structures or the brain might be. Furthermore there was no clue as to what genes this putative transcription factor might regulate, or how it might itself be regulated.

The aims of the project were two-fold: Primarily the intention was to obtain information about the roles of the *Pax6* gene in normal development, specifically what developmental processes it was important for. Secondly it was recognised that if such processes could be identified, they could themselves be analysed, by examining *Small eye* animals in which they were disrupted.

1.7.1 Experimental approach adopted

Of the approaches that could have been taken, many would greatly benefit from the basic information such as what stages of development were first affected in *Pax6* mutants and where *Pax6* was expressed. This information could, for example, narrow the search for targets and suggest ways in which *Pax6* could be regulated.

The approach taken was to characterise the normal mRNA expression pattern of the *Pax6* gene. Simultaneously, developmental defects of *Small eye* mice would be analysed. Either approach might on its own yield valuable information on *Pax6* function, but the two types of information could be combined. This might show which aspects of the expression pattern are the most significant and, conversely, which defects were in *Pax6*-expressing tissues, and which might secondary consequences of defects in other tissues. There was cause for optimism that *Pax6* would be developmentally regulated and that there would be some relationship

between the expression pattern and mutant phenotype. For *Pax6*-related genes such as *Drosophila paired* and *gooseberry*, much important information had been obtained from mutant phenotypes and expression studies (Nüsslein-Volhard and Weischaus, 1980; Sander *et al.,* 1980; Kilchherr *et al.,* 1986; Patel *et al.,* 1989; Gutjahr *et al.,* 1993). Moreover these two approaches had proved to be consistent and complementary, with defective antero-posterior patterning in mutants and segmentally repeated gene expression patterns. (Nüsslein-Volhard and Weischaus, 1980; Kilchherr *et al.,* 1986; Baumgartner *et al.,* 1987; Baumgartner and Noll, 1990) The vertebrate *Pax1* gene was developmentally regulated (Deutsch *et al.,* 1988) and its expression pattern, and the *undulated* mutant phenotype (Gruneberg, 1954) were consistent, both pointing to a role in axial skeleton development (Balling *et al.,* 1988). Most significantly, human *PAX6* was not ubiquitously expressed, and instead was found to be expressed within the eye, an affected structure in both aniridia and *Small eye* (Ton *et al.*, 1991).

1.7.2 Motivation behind the use of *Sey/Sey* embryos

It is of considerable clinical interest to know how *Pax6* mutations result in cataracts and absence of iris. In humans, this appears to occur from a 50% reduction in *PAX6* dosage (Ton *et al.,* 1991). Finding a process sensitive to *Pax6* dosage would be important, but it was considered that a clearer picture on the normal roles of *Pax6* would be obtained by examining the defects in animals with as little normal PAX6 function as possible. Deletion mutants were not used however as other, unknown, genes could be affected in these animals. Instead *Sey/Sey* animals were chosen to be to be examined.

Hogan *et al.,* (1986), found that both eye and nasal development were disrupted in *Sey/Sey* animals at, or around, the time of placode formation. This meant that roles for *Pax6* in late developing structures would be missed from the analysis of *Small eye* homozygous mutants in which they did not develop, but it also provided some specific questions to examine in the light of the new information that *Pax6* was mutated in *Small eye*: 1) How early could the lens and nasal cavity defects be detected, now that the genotype of embryos could be determined molecularly? (Hill *et al.,* 1991). 2) What was the relationship between normal *Pax6* expression and lens formation? Human *PAX6* was known to be expressed in both lens and retina (Ton *et al.,* 1991), but to address this question it would be necessary to trace this expression back prior to the time of lens placode formation. 3) Was the common origin of lens and nasal cavity defects, suggested by Hogan et al (1986), supported by common features of the *Pax6* expression in lens and nasal cavity development?

These questions provided a starting point for the central goal of the project, to determine processes in eye and nasal development that PAX6 was important for. The role of the *Pax6* gene in olfactory bulb development was not specifically studied. During the project however, earlier forebrain abnormalities were recognised in *Sey/Sey* embryos, and these were characterised to provide further information on how *Pax6* mutations could affect development.

1.7.3 Use of molecular markers for the analysis of expression patterns and phenotypes

Pax6 expression patterns were characterised using a number of region and tissue-specific gene expression patterns. Some of these markers, and others, were

used to assist in the identification of structures in *Sey/Sey* embryos. In this way, relationships between *Pax6* and other genes could be determined and the *Sey/Sey* defects could be identified as specifically as possible. *Pax6* expression itself was used as a marker in *Sey/Sey* embryos, so that sites where defects occurred could be directly related to the sites of *Pax6* expression. This study of gene expression in *Sey/Sey* embryos had the potential to reveal genes downstream of *Pax6*, if changes in gene expression were detected in the mutants. For two markers, an *Msx1* transgene and *Pax6* itself, such changes were found, thus allowing suggestions to be made about genes that might be regulated by PAX6, and also a way in which *Pax6* might be regulated.

CHAPTER 2

Materials and Methods.

Alterations to methods.

The experiments described in this thesis were spread over a period of about three years. During this time changes and improvements were made the two main techniques used, the histological methods and *in situ* hybridisation with ^{35}S riboprobes. The methods presented here are the basic techniques as they were most usually applied in this work, and are accompanied by notes of changes that were made. Some of the changes were simply ones of equipment used, but these sometimes resulted in more controllable experiments that were easier to perform.

It has not always been possible for me to personally make direct comparisons between the original and modified methods using identical starting material. Thus, there is some subjectivity here as to what constitutes an improvement in the method, and I have relied on the advice of workers who have made such comparisons. Changes to histological techniques were generally made on the advice of experienced histology practitioners, particularly Allison Ross, Duncan Davidson and Corrine Arnott. The parameters of the radioactive *in situ* hybridisation technique have been recently systematically varied by Liz Graham and Duncan Davidson, and changes to my own *in situ* technique are usually as a result of their unpublished observations.

For most of the other methods described, experiments were either performed as a single series, or at different times but using an identical method.

Methods to be described.

In this chapter some methods are described in more detail than others. Extra emphasis has been given to the histological and *in situ* hybridisation techniques, around which the project was centred. Methods that differ from commonly published

techniques, or unusual methods, not well represented in the literature, also receive more explanation than others. In particular the whole-mount *in situ* hybridisation technique used here has not been published in full. It was developed by Lesley McInnes and is an amalgam of different published and unpublished methods. The computer image processing techniques presented here are not in common usage. They have been outlined in Monaghan *et al.,* (1991) but have since changed considerably. The description given here is intended to be a more up-to-date practical guide to using these methods.

2.1 *Small eye* alleles and genetic background.

For all the core experiments in this study the allele of *Small eye* used was *Sey* (Lyon and Searle, 1989), in which a G to T transversion produces a stop codon in the translated sequence prior to the PAX6 homeodomain (Hill *et al.,* 1991). In an initial histological study, and in the study describing eyelid development (see results), Sey^{Neu} homozygous mutants embryos were also used. In this allele (the ENU 2029 mutation produced by Jack Favor), a G to T transversion at a splice donor site results in aberrant splicing of the 3' end of the *Pax6* gene, predicted to replace the C-terminal 115 amino acids by a short (22 amino acid) intron-encoded peptide (Hill *et al.,* 1991).

Sey animals, selected (by animal house staff) on the basis of eye size, were maintained on a CBA (inbred, pigmented) background and also by repeated outcrossing with (non-pigmented) Swiss animals. Sey^{Neu} embryos analysed had a (102 × C3H)F1 hybrid background. *Msx1* transgenic animals were of mixed genetic

background as transgenics were initially produced on a (CBA × C57BL/6)F1 background and were ultimately mated to Swiss *Sey* animals, producing males that were further mated to Swiss or CBA *Sey* females.

2.2 Choice of experimental and control animals.

Non-pigmented Swiss mice were used to prevent artefactual signal from melanin pigment in *in situ* hybridisation experiments. Other experiments used either the pigmented or the non-pigmented animals described above, but control animals and *Sey/Sey* animals had comparable genetic backgrounds, either by being littermates, or by drawing control embryos from the appropriate Swiss or CBA colony.

Embryonic development is assumed to have begun at midnight of the night of mating determined by the presence of a vaginal plug the next morning. Homozygous *Small eye* mice die at birth, (Hogan *et al.,* 1986), thus the *Sey/Sey* embryos used were obtained from crosses of heterozygous *Small eye* parents. Littermates from these matings, (*Sey/+* and +/+ embryos), provided control embryos of matched developmental stage.

In the majority of cases where comparisons between *Sey/Sey* embryos and their littermates are made, the littermates have also been compared with wild-type embryos derived from wild-type parents. For expression of *Tyrp2* and *Pax6* and for programmed cell death studies, this comparison was based on my own studies and those of other lab members (Ian Jackson, Duncan Davidson, Liz Graham, and Bob Hill) supplemented by published data for wild-type mice (Steel *et al.,* 1992; Walther

and Gruss, 1991; Sulik *et al.,* 1981). For *Msx1* transgene expression, information on the normal pattern of expression in this line comes from the unpublished observations of Duncan Davidson. For *entactin* expression, expression seen in littermate controls was compared with published wild-type patterns (Dong and Chung, 1991). Comparisons were not made between littermate controls and wild-type embryos for the electron microscopy studies.

2.3 Sample sizes and dealing with small sample size.

Table 2.1 gives an indication of the numbers of specimens analysed in each type of experiment, divided between *Sey/Sey* and control embryos where appropriate. I have chosen to list numbers of embryos examined and not numbers of organs or numbers of sections. This is mainly for simplicity and consistency but also so that the numbers of samples are not overestimated. The number of eyes and nasal cavities examined are both usually twice the figure given for numbers of embryos.

Some of the smaller numbers in this table require comment. Three categories can be identified. Firstly there are experiments involving sections, where the number of embryos used is small but the number of sections is much larger (usually several hundred). Secondly, there are experiments where the number of embryos listed is small but the combined numbers that formed part of this study was larger, as certain categories of embryos have been excluded from the figures. Thirdly there are cases where a small number of embryos does not represent a larger number of sections or of embryos examined.

Care has been taken in drawing up Table 2.1 not to over-represent any figure, but many of the values will under-represent the numbers of embryos examined for

several reasons; 1) Histological information was also gained from embryos on which *in situ* hybridisation had been performed, but the two figures are listed separately. 2) The figures exclude isolated successful *in situ* hybridisation sections in largely unsuccessful experiments, although the results from these sections supported the results obtained from more comprehensive series of sections in successful experiments. 3) In the case of Nile Blue Sulphate staining, where patterns diffuse quite rapidly, all embryos in the litters were stained and examined but only representative embryos were photographed. Other figures for whole mount embryos similarly reflect those that were photographed, although larger numbers were prepared and viewed.

Small numbers of embryos reflect small numbers of samples analysed mainly in the electron microscopy studies. The principal conclusions in this case concern the formation of the placode and its contact with the optic vesicle, and are fully consistent with the results of high power light microscopy. Other results, using the electron microscope at higher magnifications, are presented as interesting phenomena that may merit further investigation.

TABLE 2.1

Numbers of embryos used in eye and nasal development study.

Study	Age (E)	*Sey/Sey*	Controls Total(WT)
Semi-thin sections and TEM	9.5-10	2	2
Haemotoxylin and eosin histology	9.5-9.75	3	?
	10-10.75	?	?
	11-11.75	3	1+?
	12.5	5	1
	13.5	1	1(1)
	14.5	3	2(2)
	15.5	2	2(2)
***Pax6* mRNA expression**	8.0	2	5(4)
	8.5-8.75	2	5(3)
	9-9.5	4	8(6)
	9.75-10.25	3	6(2)
	10.25-11.25	1	9(8)
	11.5-12.5	7	8(3)
	14.5-15.5	1	1(1)
	16.5-17.5	0	2
***Tyrp2* and *entactin* mRNA expression**	11.5-12.5	2	4(nd)
***Msx1* transgenics**	9.5-9.75	4	9
	10.5	2	9
	11.5	4	4
Nile Blue Sulphate detection of cell death	9-9.75	2	12(8)
	10.5	2	8(8)
	11.5	3	8(8)

2.4 PCR genotyping of young embryos

The *Sey* mutation (Hill *et al.,* 1991) creates a diagnostic *Dde*I site that allows embryonic material to be genotyped by PCR amplification and *Dde*I digestion. For genotyping, paraformaldehyde fixed embryos were bisected using tungsten needles in PBS at the level of the midgut (8 day) or heart (8.5 day and later embryos). Anterior portions were aligned a few millimetres apart and embedded in a 3% low melting point agarose gel made with PBS. The agarose block was cut in such a way that each embryo could later be unambiguously identified and then fixed in 4% PFA, processed routinely for histology and embedded in wax as described below. In this way similar sections of embryos of different genotypes appear on the same slide and are hybridised equivalently. Posterior embryo parts were individually washed twice in PBS then digested in 20 µl with 6 µg/ml proteinase K for 1 hour at 55°C in 1× PCR buffer, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.1 mg/ml gelatine and 0.1% Triton X-100, with added non-ionic detergents, 0.4% Tween 20 and 0.4% NP40. Proteinase K was inactivated at 95°C for 10 minutes.

PCR reactions and the analysis of the products were performed by Bob Hill. 1-2 µl of digested sample was taken for each PCR reaction. PCR reactions were performed in total volumes of 25 µl using 1 pmole/ reaction of each primer B509 (TGCCAGCAACAGGAAGGAGG) and E723 (CTTTCTCCAGAGCCTCAATCTG) with 0.2mM of each dNTP in the 1 × PCR buffer described above. Taq polymerase (1 unit/ reaction) was added after an initial denaturing step of 4 minutes at 95°C. 35 cycles (95°C for 30 seconds, 61°C for 30

seconds and 72°C for 48 seconds) were performed followed by a single extension step of 72°C for 5 minutes. Following phenol-chloroform extraction and ethanol precipitation, samples of PCR products were digested with *Dde*I. The 154 bp PCR product was cut to give 83 bp and 71 bp fragments for wild type alleles and 83 bp, 51 bp and 20 bp fragments for *Sey* alleles. These digestion products were analysed on 4% NuSieve GTG agarose gels (FMC) in 1 × TBE buffer.

2.5 Processing of tissue for *in situ* hybridisation

As with all methods involving embryos, pregnant females were killed by cervical dislocation, and embryos were collected into ice cold phosphate buffered saline(PBS). For radioactive *in situ* hybridisation, embryos were fixed overnight in 4% paraformaldehyde (PFA, pH 7.2) in PBS at 4°C. To embed the embryos in parafin wax several steps are required: 1) Removal of fixative by washing in PBS. 2) Dehydration through a graded series of alcohols. 3) Clearing of embryos. 4) Impregnation of 56°C melting temperature wax. 5) Orientation of the embryo and solidifying the wax. Two main methods were used for embedding which differed at most of these steps. The principal differences were: 1) The choice of solvent for clearing, which was changed from histoclear to xylene when a sealed unit became available that would minimise exposure to toxic xylene fumes. 2) The impregnation of wax was first performed at atmospheric pressure. In the later method, vacuum was used to remove the previous fluid (alcohol, xylene or wax) and pressure was used to assist with the penetration of the new fluid. 3) The apparatus used for the producing a block was considerably altered.

There were also some differences in the length of time embryos spent in each fluid. Three procedures used are described below. The first two are the main methods used with the differences outlined above. The first method uses histoclear, the second uses xylene. The third method described was adopted for young embryos and uses the apparatus of first method, but with xylene as the clearing agent.

2.5.1 Wax embedding for PFA-fixed tissue, clearing in histoclear.

The steps and times for each step in this procedure are shown in Table 2.2. In this method all changes of solutions were performed by moving the embryo from solution to solution in a 24 well plate using a broad gauge sterile pipette (typical for 9-10 day embryos or 11.5 day heads) or by draining solution form the embryos and replacing it with fresh solution. Draining the solutions from dissected heads, occasionally resulted in air bubbles being trapped in the brain ventricles, giving poor embedding and sectioning properties. Conversely damage to embryos appeared to occur more frequently when embryos rather than solutions were being transferred. This damage could be reduced by using glass pipettes to which embryonic material was less likely to stick.

Where embryos were processed individually volumes of solutions were typically 1.5- 2 ml in a 24-well plate or 4 ml in a 5 ml Sterilin tube. Litters of embryos processed together used 4 ml at 8.5-9.5 days and 20-40 ml for older embryos. Glass sample tubes (10-15 ml) and glass pipettes were used for histoclear.

Wax baths were in watchglasses in an 60^{o}C oven. Transfer of embryos from one wax bath to another proved problematic. A heated glass pipette or metal spoon spatula were used but these was suspected to overheat the embryo. Other methods

were thus employed. Passing 60°C wax in and out of a previously heated glass pipette to equilibrate the temperature, or keeping and transferring embryos in a fine metal basket (Tissue Tek III biopsy box, Bayer Diagnostics), were found to be convenient methods.

The final embedding was in a solid glass dish coated with a thin layer of glycerol to ease removal of the block. Orientation and embedding was performed by manipulating the embryos using flame heated forceps and viewing under a dissection microscope. Once embryos were firmly positioned in solidifying wax, dishes were placed on ice to rapidly set wax, before being submerged in cold water overnight.

TABLE 2.2

Processing material for *in situ* hybridisation, clearing in histoclear.

Solution	Time E8.0 - E9.5	Time - E 10 -E13.5	Temperature
4% PFA in PBS	overnight	overnight	4°C
PBS	2 × 5 min	2 × 15 min	4°C
0.85% NaCl	5 min	30 min	4°C
1:1 0.85% NaCl, ethanol	5 min	15 min	4°C
70% ethanol	5 min	15 min	R/T
70% ethanol	5 min	30 min	R/T
85% ethanol	5 min	30-60 min	R/T
95% ethanol	5 min	1 hour	R/T
100% ethanol	3 × 15 min	3 × 1 hour	R/T
Histoclear	2 × 15 min	2 × 30 min	R/T
Histoclear	1- 2 hour	overnight	R/T
56°C melting temperature paraffin wax	3 × 30 min	3 × 60 min	60°C

R/T, room temperature

2.5.2 Wax embedding for PFA-fixed tissue, clearing in xylene

This method, used for embryos from E10.5 onwards, employed a Tissue Tek VIP 1000 vacuum infiltration processor (Miles Scientific). Solutions in this machine were not sterile, and so processing to 70% ethanol was performed by hand with sterile solutions if blocks were required for *in situ* hybridisation. Two programmes for the automated tissue processor were devised, one for embryos up to E11.5 and another for older embryos. These are shown in Table 2.3 and Table 2.4 respectively. The principal differences are the times for each step, but pressure and vacuum was thought to be unnecessary for the younger embryos as was omitted until the last 100% ethanol step.

The final embedding procedure was also different. Wax for embedding was kept at 60°C in a Tissue Tek thermal console (Miles Scientific) into which the biopsy cassettes containing embryos were transferred. This device also maintained the metal moulds for forming blocks, and forceps for manipulating embryos, at the same temperature. The metal moulds cooled too rapidly for embedding under the dissection microscope. Instead they were placed on a hot plate set slightly lower than the melting temperature of the wax, giving more time to position the embryos. Once wax around the embryos had set, the external surface of the mould was cleaned of surplus wax to allow uniform temperature transfer, and the moulds were placed on melting ice until the block could be released.

TABLE 2.3

Procedure for embedding embryos E10.5 and E11.5 using vacuum.

Solution	**Time**	**Temperature**	**Pressure / Vacuum**
4% PFA in PBS, pH 7.2	O/N	0-4 °C	-
Phosphate Buffered Saline	2 × 15 min	0-4 °C	-
0.85% NaCl	15 min	0-4 °C	-
1:1 O.85% NaCL:Ethanol	15 min	0-4 °C	-
50% Ethanol	15 min	0-4 °C	-
70% Ethanol	15 min	room temp	-
70% Ethanol (in processor)	variable	40 °C	NO
85% Ethanol	20 min	40 °C	NO
95% Ethanol	20 min	40 °C	NO
100% Ethanol	20 min	40 °C	NO
100% Ethanol	20 min	40 °C	YES
Xylene	2 × 20 min	40 °C	YES
Wax	3× 30 min	60 °C	YES
Wax	embedded	60 °C	N/A

TABLE 2.4

Procedure for embedding embryos E12.5 - E15.5 using vacuum.

Solution	**Time**	**Temperature**	**Pressure / Vacuum**
4% PFA in PBS, pH 7.2	O/N	0-4 °C	-
Phosphate Buffered Saline	2 × 30 min	0-4 °C	-
0.85% NaCl	30 min	0-4 °C	-
1:1 O.85% NaCL:Ethanol	30 min	0-4 °C	-
50% Ethanol	30 min	0-4 °C	-
70% Ethanol	30 min	room temp.	-
70% Ethanol (in processor)	variable	40 °C	YES
85% Ethanol	60 min	40 °C	YES
95% Ethanol	60 min	40 °C	YES
100% Ethanol	2 × 60 min	40 °C	YES
Xylene	2 × 40 min	40 °C *	YES
Wax	3 × 60 min	60 °C	YES
Wax	embedded	60 °C	N/A

* Second xylene later changed to 50°C as excessive temperature change was suspected of causing poor wax penetration

2.5.3 Embedding small PFA fixed embryos using xylene

This method, set out in Table 2.5, was used for the more delicate embryos, from 8 to 9.75 days gestation. The younger embryos in this range were kept in each fluid for the shorter of the times shown in the table. The longer times shown are those appropriate for E9-E9.75 embryos. The principal stages are as for other methods, but a 50% ethanol:50% xylene stage was included. The equipment was as for the method using histoclear.

TABLE 2.5

Hand embedding of small embryos (E8.0-E9.75) using xylene.

Solution	Time	Temperature
4% PFA in PBS, pH 7.2	O/N	0-4 °C
Phosphate Buffered Saline	2 ×10 min	0-4 °C
0.85% NaCl	5-10 min	0-4 °C
1:1 O.85% NaCL:Ethanol	5-10 min	0-4 °C
50% Ethanol	5-10 min	0-4 °C
70% Ethanol	5-10 min	room temp
70% Ethanol	variable/store	room temp
80% Ethanol	5-10 min	room temp
90% Ethanol	5-10 min	room temp
100% Ethanol	2 × 5-10 min	room temp
50% Ethanol: 50% Xylene	5-10 min	room temp
Xylene	until clear	room temp
Wax	3 × 10 min	60 °C
Wax	embedded	60 °C

2.6 Histology techniques.

2.6.1 Embedding of Bouin's-fixed material.

Material exclusively for histology was fixed in Bouin's fixative at room temperature. This fixative is a 75:25:5 by volume mixture of saturated aqueous picric acid solution, 40% formaldehyde and glacial acetic acid. Fixation times were from 2-8 hours (E9-9.75, 2 hr; E10-11.75, 3 hr; E12-13.75, 4 hr; E14.5, 5 hr ; E15.5 6 hr; E17.5, 8 hr). Following fixation in Bouin's, embryos were transferred directly to 70% ethanol, and then either processed to wax or placed in fresh 70% ethanol and stored until required. The time in each solution was varied depending upon the age of the embryo according to the protocol of Corrine Arnott, shown in Table 2.5. The apparatus was mainly the same as for PFA-fixed tissue, with use of the vacuum embedding device dictated by the size of embryo. An alternative set-up was also used differing principally at the wax embedding stage. In this alternative set-up, molten wax was kept in metal moulds and maintained at the correct temperature by placing a strong heat lamp above the work surface. Forceps were also heated by this lamp rather than by a flame, reducing heat damage to tissue.

TABLE 2.6

Embedding mouse embryonic material following fixation in Bouin's fixative. (Method of Corrine Arnott)

		SOLUTIONS								
		Ethanol series					Xylene	60°C Wax Baths		
		70% *+	96%a	96%b	100%a	100%b		a	b	c
E A	Day 11	>7 min	7 min	7 min	7 min	7 min	U	7 min	7 min	7 min
M G	Day 12	>12 min	12 min	12 min	15 min	15 min	N C	15 min	15 min	15 min
B E	Day 13	> 20min	20 min	20 min	20 min	20 min	T L	20 min	20 min	20 min
R	Day 14	>30 min	30 min	30 min	30 min	30 min	I E	20 min	20 min	20 min
Y	Day 15	> 40 min	40 min	30 min	40 min	30 min	L A	20 min#	20 min#	20 min#
O	Day 16	> 45 min	45 min	45 min	45 min	45 min	L R	30 min #	30 min#	30 min#
	Day 17	> 60 min	60 min	45 min	60 min	45 min		45 min#	45 min#	45 min#

+ Store in 70% following fixation.

* Several 70% washes may be required to help remove fixative.

Use vacuum embedding.

2.6.2 Wax sections for haemotoxylin and eosin histology

Slides were either untreated, acid and alcohol washed or washed and coated with a thin layer of glycerol albumin. For acid washing, slides in glass racks were dipped for 10 seconds per solution in 10% HCl:70% ethanol, in distilled H_20, and then in 95% ethanol. Washed slides were then baked for 1 hr at 150-180°C and allowed to cool. The glycerol albumin was 50% glycerol, 1.5% bovine serum albumin with 1µl/ml of phenol as a preservative, and was stored at 4°C. Wax sections were cut to a thickness of 6-8µm, typically 6µm, on a Reichert-Jung Biocut 2030 microtome with Tissue-Tek III Accu-Edge microtome blades. Sections were floated onto slides either from a waterbath at 45°C, or on a hotplate at the same temperature until stretched. Glycerol albumin coated slides were only used with the hotplate. Sections were baked onto slides at 50-60°C overnight.

2.6.3 Haemotoxylin and eosin histology

Slides were dehydrated through an ethanol series (5 minutes each in 30%, 50%, 70%, 90% and 2 × 100% ethanol), and wax was removed by two changes of xylene for five to ten minutes each. Slides were then rehydrated through the ethanol series and placed in tap water or several minutes, before being placed in Harriss' haemotoxylin for four minutes with occasional gentle agitation. Stained slides were removed into running tap water and thoroughly washed. Differentiation of the haemotoxylin was performed by immersing in acid alcohol (1% HCl in 70% ethanol) with continuous agitation for a few seconds (8-15 seconds, typically 10) . The

haemotoxylin was blued up for several minutes in Scott's tap water, consisting of 0.2% (w/v) $KHCO_3$ and 2% (w/v) $MgSO_4.7H20$. The slides were washed well in running tap water and transferred to 1% aqueous eosin to stain for five minutes with occasional gentle agitation. Stain retention was improved if acetic acid was added to the eosin immediately before use to give a 0.05% final concentration. Eosin stained slides were very briefly rinsed in tap water and transferred to 100% ethanol. A brief intermediate 50% ethanol wash was occasionally used. After one to two minutes in 100% ethanol slides were transferred to fresh 100% ethanol for a further one to two minutes. Test slides, covered with 100% ethanol, were viewed under a dissection microscope and slides were returned to 1% eosin solution if the eosin staining was insufficient. Well stained slides were thoroughly dried in air (or in a 60°C oven), cleared in xylene for one to two minutes, and mounted in DPX.

2.6.4 Semi-thin sections

Semi-thin sections for histology were produced by dehydrating fixed E11.5 heads and E14.5 brains through a series of alcohols and embedded in LR white (London Resin Company) using accelerator according to the manufacturers protocol. Sections were cut at 2μm with a glass knife, floated out onto slides on 40% acetone, 60% boiled distilled water on a hotplate at 65-75°C, then heated over a flame to bake to the slide and stained with 0.1% Toluidine Blue, 0.1% Borax in 70% ethanol.

2.7 *In situ* hybridisation with ^{35}S riboprobes.

2.7.1 Control probes and templates for *in situ* hybridisation probes.

Positive control probes were selected such that expression would be present on most sections, but not in all tissues, thereby also serving as negative (no hybridisation) controls. *Msx1* control probe (Monaghan et al ,1991) detecting a transcript with known expression pattern (Hill *et al.,* 1989; Mackenzie *et al.,* 1991a,b; Monaghan et al ,1991) was used to in this way. Later *Msx1* expression in the brain is largely restricted to the developing choroid plexus (Mackenzie *et al.,* 1991b) thus for the positive control in neural tissue a probe was used detecting transcripts of the LIM-like homeobox gene *LH-2* (Xu *et al.,* 1992b), which is strongly expressed in specific domains of the brain but has little overlap in expression with *Pax6*. In the eye the *entactin* gene provided a useful control probe for the analysis of the optic vesicle, as its mRNA is not expressed in this tissue.

A gene-specific 1 kb template for making mouse *Pax6* RNA probe was made by polymerase chain reaction, PCR, from a 1.6 kb cDNA clone, pm1 (Ton *et al.,* 1991), using oligonucleotide C128 (ATGGTTTTCTAATCGAAGGG) from the 3' end of the *Pax6* homeobox, and a T7 promoter oligonucleotide (AATACGACTCACTATAG) matching vector sequence. A similar procedure was used to make the *entactin* and *LH2* probe templates. For *entactin* oligonucleotides D932 (CAGTGTCACCACAGCACTGGC) and T3 promoter oligonucleotide (ATTAACCCTCACTAAAG) were used to amplify a 1.6 kb region at the 3' end of clone p633, a gift of Prof. Albert E. Chung. For *LH2*, template was produced by PCR using T7 and T3 (ATTAACCCTCAAAG) oligonucleotides from plasmid BR2

containing the 600 bp *Bgl*II-*Eco*RI fragment of the rat *LH-2* gene, used by Xu *et al.*, (1992b), to study expression of the highly homologous mouse gene.

In all cases the both cases reactions were in total volumes of 50µl with a 1 × PCR buffer consisting of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.1 mg/ml gelatine and 0.1% Triton X-100.The dNTPs were used at a final concentration of 0.2mM each. 25 pmoles/reaction of each oligonucleotide was used and the starting templates for PCR reactions were 1-5 ng of plasmid DNA. Taq polymerase (5 unit/ reaction) was added after an initial denaturing step of 4 minutes at 95°C. 30 cycles (95°C for 30 seconds, 56°C for 30 seconds and 72°C for 90 seconds) were performed followed by a single extension step of 72°C for 5 minutes. PCR product was quantified by running 1µl of reaction product on a 1% agarose gel in 1 × TBE buffer alongside molecular size markers of known concentration. Specificity of the reaction product was tested by running 10µl of the negative control reaction, that lacked the DNA template, on these gels. PCR product was extracted by phenol-chloroform followed by ethanol precipitation before being transcribed.

The template for the *Tyrp2* probe, a gift of Ian Jackson, was the 1.2 kb clone p5A7 described in Steel *et al.,* (1992), digested with *Hind* III. The template for the s*teel* probe, also a gift of Ian Jackson, was the Bluescript vector containing the 467 bp *Nsi*I to *Sac*I fragment described in Steel *et al.,* (1992), which was digested with *Hind*III.

2.7.2 *In vitro* transcription reactions for ^{35}S riboprobes

^{35}S labelling reactions were performed in a total volume of 30µl or 60µl adding each chemical in a set order. Enzymes were kept at -20°C, except whilst being dispensed, when they were placed on ice. All other chemicals were allowed to reach room temperature before use. For a 30µl reaction the starting point was 6µl of 5 × transcription buffer (5 × buffer is 200mM Tris-HCl; 30mM MgCl2; 50mM dithiothritol (DTT); 10mM spermidine, pH 8.0) to which 1µl of 10mM rATP, rCTP and rGTP (final concentration 333 µM each), 1µl of 1M DTT, and 3µl sterile (autoclaved) distilled H_20 were added. The ^{35}S rUTP used had a specific activity greater than 1mCi/100µl, and was used at 12µl per 30µl reaction. DNA templates were added in a volume of 5µl. For a 3 kb plasmid with a 1 kb insert, 500 ng to 1µg of template DNA was used. Where DNA templates were PCR products, they were used at equivalent molar concentrations, i.e. 125-250 ng for 1 kb products. 1.2µl RNasin (RNase inhibitor)(12-60 units) and 0.8µl of the appropriate RNA polymerase (T7, T3 or SP6; Boehringer) (20-40 or 10-20 units/µl) were added and mixed well and the reaction mixtures were spun down briefly prior to an incubation at 37°C for 25 minutes. After this time fresh RNase inhibitor and RNA polymerase were added, mixtures were spun down briefly again and incubated for a further 25 minutes at 37°C. 20 µg of carrier RNA (tRNA or rRNA) was added, and DNA template was digested by adding 1µl (20 -50 units) of DNase I (RNase free) and incubating at 37°C for 10 minutes. 2µl of 100mM EDTA was added then the volume of the reaction mix was made up to 200 µl with TE buffer with 50mM DTT. Probes

were extracted first with an equal volume of phenol, then with 25:24:1 mixture of phenol, chloroform and isoamyl alcohol and then with chloroform, and were then precipitated overnight at -20°C in 75% ethanol, 0.3M ammonium acetate pH 5.2. RNA was pelleted by spinning for a total of 30 minutes at 12,500g . Pellets were washed with cold (-20°C) ethanols, first twice with 1 ml of 80% ethanol: 50mM DTT then once with 100% ethanol. To minimise aerosol contamination, pellets were dried by placing tubes covered in pierced parafilm in a heated vacuum centrifuge for 10 minutes (or until dry). Pellets to be used without alkali hydrolysis digestion were resuspened in 20µl of sterile distilled H_20 with 50mM DTT and stored at -70°C. If probes were to be digested (see below), 100µl of the same solution was used.

2.7.3 Partial hydrolysis of riboprobes.

To improve probe accessibility, probes over 1 kb in length were fragmented by partial alkali hydrolysis. This was done by incubating the probe in carbonate buffer pH 10.2 with 33mM DTT at 60°C for a time, t (typically between 40 and 70 minutes) calculated according to the formula :

$$t = \frac{(\text{start length, kb}) - (\text{desired length, kb})}{(K)(\text{start length, kb})(\text{desired length, kb})}$$

K is the rate constant 0.11. The desired average length is about 0.15 kb or slightly greater. For a 1.6 kb starting probe this gives a time of 55 minutes. The 2 × carbonate buffer is a 3:2 mixture of 0.2M Na_2CO_3 and 0.2M $NaHCO_3$. Digestion is stopped by adding 1/3 volume of 3M ammonium acetate pH 5.2 and probe is precipitated as described above with 3 volumes of 100% ethanol at -20°C overnight.

2.7.4 Determining the specific activity of ^{35}S labelled riboprobes

1µl of each probe to be used was diluted with 19µl of TE buffer and 8µl of diluted probe was spotted onto each of two Whatman GF/B glass fiber filters. One of these filters was washed with 3 × 10-15 ml, 5% trichloroacetic acid (TCA) at 4°C, and then with 15 ml of 100% ethanol , 4°C and allowed to dry. When both filters were dry they were transferred to scintillation vials with 10 ml Aquasol-2 and counted in a scintillation counter. The ratio of the measurements from the washed and unwashed filters gave an indication of the level of incorporation of label. From the activity measured from the washed filter the specific activity of the probe was calculated assuming a counting efficiency of 50%, i.e. that one count per minute (cpm) represents to two decays per minute (dpm). All probes were used at an activity of 1×10^5 dpm/µl, and were thus diluted to 1×10^6 dpm/µl before adding to the hybridisation mix.

2.7.5 Pre-treatment of slides for *in situ* hybridisation

Microscope slides for *in situ* hybridisation were washed and coated with TESPA (3-aminopropyltrimethoxysilane, Sigma Chemical Company) to prevent sections from being removed during the *in situ* procedure. Slides in glass racks were dipped for 10 seconds per solution in 10% HCl:70% ethanol, in distilled H_20, and then in 95% ethanol. Washed slides were then baked for 1 hr at 150-180°C and allowed to cool. The coating was performed for at least 10 seconds using 2% TESPA freshly made up in acetone and well mixed. Excess TESPA was removed using two washes, of 10 seconds each, in 100% acetone and slides were then washed for 10

seconds in sterile (autoclaved) distilled H_20, and dried at room temperature or in an oven at 42°C. Slides were continuously agitated in all solutions to ensure the whole slide was treated, and racks of slides were briefly drained between solutions to minimise carry-over from one solution to another. Solutions were changed after each batch of 5-8 racks. Slides were stored desiccated for up to 7 days. Care was taken to ensure that prepared slides were handled only with sterile forceps or with ethanol washed gloves to minimise contamination by RNase and glove chalk.

2.7.6 Pre treatment of coverslips for *in situ* hybridisation

Coverslips for the hybridisation step of *in situ* hybridisation were washed briefly in sterile distilled H_20 to remove any dust then were soaked twice for ten minutes each in 100% ethanol and allowed to dry in air on fresh lint free tissues, handling with clean forceps they were cut to size if necessary using a diamond pen, and were used immediately.

2.7.7 Preparation of sections

Wax sections were cut to a thickness of 6-8µm, typically 7µm, on a Reichert-Jung Biocut 2030 microtome with Tissue-Tek III Accu-Edge microtome blades. Steps were taken to avoid RNase contamination of sections and to ensure minimal distortion during cutting. Grease on new blades and fragments of wax on the microtome, and on used blades were removed by cleaning the microtome surface and blades with xylene and then ethanol before use. All tools used for handling sections were cleaned with ethanol and blowing on sections, commonly employed for histological sections, was avoided when sections were for *in situ* hybridisation.

Sections were floated at 45°C onto TESPA coated slides in an alcohol-washed waterbath containing sterile (autoclaved) distilled water. Gloves were worn for handling slides, but were cleaned to remove chalk which might contaminate slides giving non-specific signal. Sections were baked onto slides at 60°C overnight and then desiccated under vacuum and stored in a sealed box with desiccant at room temperature until required.

2.7.8 Pre-hybridisation treatment of sections

Prior to the pre-hybridisation procedure slides were desiccated again overnight under vacuum to ensure that they were totally dry. Wax was removed by soaking in two baths of xylene for ten minutes each. Two 100% ethanol washes with gentle agitation for two minutes were used to remove xylene.

Slides were rehydrated though a series of 90%, 70%, 50% and 30% ethanol for 2 minutes each followed by 0.85% NaCl for five minutes and PBS for five minutes. The sections were fixed to the slide by immersion in 4% paraformaldehyde (PFA) in PBS pH 7.2 for twenty minutes. To remove fixative that might inhibit the proteinase K step, slides were washed twice for five minutes in PBS. The first of these washes was with separated, rather than back-to-back, slides to ensure fixative did not remain between slides. Proteinase K digestion, to improve access of RNA probes to mRNA in the section, was performed for 7.5 minutes using 20mg/ml proteinase K in 50mM Tris, 5mM EDTA pH 7.2. Excess proteinase K solution was drained off and slides were washed in PBS for five minutes. The proteinase-digested sections were re-fixed to the slide using 4% PFA in PBS pH 7.2 for five minutes and

fix was removed with a five minute wash in PBS. Slides were rapidly immersed in distilled H_20 to remove the phosphate buffer and then equilibrated in a buffer of 0.1 M triethanolamine pH 8.0 (TEA) for thirty seconds. To acetylate basic groups on the slide and sections that would bind RNA probe non-specifically slides were twice transferred, for ten minutes each, to fresh vigorously stirred 0.1 M TEA pH 8.0 to which 625µl/ 200 ml acetic anhydride was added. Following acetylation slides were washed in PBS for 5 minutes, then in 0.85% NaCl for five minutes and were dehydrated through the ethanol series (30%,50%,70%,90%) for one minute each before being finally dehydrated in three baths of 100% ethanol for five minutes each, and air dried.

The PBS, 0.85% NaCl, distilled H_20 and 0.1M TEA and proteinase K buffer were all used as sterile solutions, replaced after each rack of slides. Paraformaldehyde was made up fresh or the night before and stored at 4°C, and was replaced after two racks of slides. PBS, 0.85% NaCl, distilled H_20 and fixative were used cold (0-4°C). Proteinase K digestion, acetylation and xylene and ethanol steps were at room temperature. Ethanol series was made up with sterile distilled H_2O and used no more than six times.

Modifications: 1) Since slides were processed back-to-back, to conserve solutions and reduce the time taken for the procedure, it was occasionally found that xylene remained trapped between slides and so this procedure was later modified to separate slides for the second 100% ethanol step and including a third 100% ethanol wash.

2) The 0.85% NaCl step following the initial rehydration of the sections was later omitted.

2.7.9 Preparation and usage of hybridisation mix

The hybridisation mix consisted of 50% formamide, 10% dextran sulphate, 1 × Denhart's, 20mM Tris HCl pH 8.0, 0.3M NaCl, 5mM EDTA, 10mM sodium phosphate buffer pH 8.0, 0.5 mg/ml RNA (yeast RNA or tRNA). Viscose solutions, particularly dextran sulphate were pipetted slowly with cut-off tips (tips with larger bore end) to reduce pipetting errors. Hybridisation mix was made up in advance and stored at -70°C. On defrosting the hybridisation mix was commonly thoroughly mixed by vortexing, and then degassed under vacuum to remove the bubbles introduced in mixing. Immediately prior to use DTT (dithiothreitol) was added to 50mM then diluted probe was added at a ratio of 1:9 of probe: hybridisation mix +DTT, and mixed by repeated pipetting. For each group of 4-6 slides, probes was heated to 80°C for 2-5 min then placed on ice during dispensing, with denature and freeze steps repeated for next group of slides. The volumes of probe in hybridisation mix were the minimum that would comfortably cover the sections. These were typically 9-11µl per half square coverslip (11 mm × 22 mm) increasing to 60-70µl per 40 mm × 22 mm coverslip.

2.7.10 Hybridisation of slides with ^{35}S riboprobes

Sections were covered by coverslips, avoiding bubbles by lowering slowly onto hybridisation mix. Care was taken to ensure that two adjacent probes on a slide

would not mix. Sections were typically spaced so as to allow several millimetres between coverslips.

To prevent drying, slides were placed in a closed box containing a tissue soaked in 5-10 mls of 50% formamide, 5 × SSC. Slides were kept upright as horizontal as possible to avoid mixing of probe or movement of coverslips. Once twenty slides were in box, it was sealed with PVC insulation tape, and then heat-sealed into two thick polythene bags. The effectiveness of the seal was tested by gently squeezing the bags once the seal had cooled. Slides were hybridised by submerging sealed bags in a waterbath at 55°C for 16-18 hours.

2.7.11 Post-hybridisation washes

Coverslips were removed by a rapid bath of 5 × SSC, 55°C, then 5 × SSC, 10mM DTT, 55°C for 15-30 minutes. The high stringency wash step was 50% formamide, 2 × SSC, 0.1M DTT at 65-68°C, (usually 68°C) for all probes used. The aim was to have 20 minutes at the wash temperature, and to avoid slides going over the desired temperature. Wash temperatures were monitored by thermometer and the waterbath was kept at most 2°C above the ideal temperature. Three 10 minute washes were performed in NTE buffer (0.5M NaCl, 10mM Tris , 5mM EDTA pH 7.5) at 37°C. Non-hybridised probe was digested for 30 min with RNase A at 40 mg/ml in NTE buffer (30 ml/ ten slides in plastic coplin jars). Slides then received an additional NTE wash for 10 minutes before a second high stringency wash, for 20 minutes as before. Unbound probe was removed by three washes of 10 minutes each in 2 × SSC, followed by three washes of 10 minutes each in 0.1 × SSC. Slides were

dehydrated for one minute each through a freshly made up ethanol series, 30%, 50%, 70%, and 90% each containing 0.3M ammonium acetate. The ammonium acetate stabilises the mRNA/ probe duplexes in these solutions. Final dehydration was in 100% ethanol, twice for five minutes each, followed by air drying.

Modifications: 1) Later, DTT was also added to the initial coverslip removal step, so that DTT was present in all high temperature steps. 2) As DTT is expensive high stringency washes were performed in small volumes (30 ml/ ten slides) in plastic coplin jars. These are poor heat conductors and wash solution would take excessive time to reach the desired temperature. These containers were replaced by small glass dishes, (100 ml/ 20 slides) with better thermal properties and DTT concentration was reduced by 50% (to 0.05M). 3) A thermocouple device was used that could be inserted into wash dishes allowed closer temperature monitoring. 4) NTE washes were later changed to 5 minutes each (from 10 minutes each).

2.7.12 Autoradiography

Hybridised slides were coated in a 33% solution of Ilford K5 emulsion at 40-42°C. All procedures using this emulsion were performed under dark safelight conditions. The single S902 safelight used was placed well away from the work area, pointed away from the work and had a sheet of dark paper placed several centimetres in front of it to further reduce the illumination. The work area was further shielded from the light source

7 ml per 10 slides of emulsion was melted in a waterbath at 40°C and mixed gently with two parts of distilled water prewarmed to 40°C. Slides were dipped back-to-back into this mixture in a dipping bath. Slides were allowed to dry overnight by placing at room temperature in a light tight box with silica gel. They were then transferred to a slide box with fresh silica gel and the box was sealed, kept dark by wrapped in two layers of aluminium foil, and stored to expose at 4°C. Exposure times ranged from 2½ to 6 weeks, depending upon the results of a few test slides from the experiment developed after 2½ to 3 weeks.

For the developing, developer, distilled water and fixative were used at around 20°C, with variation in temperature kept to a minimum, (at most 1-2°C). Under the safelight conditions described above slides in glass racks were immersed first in Kodak D19 developer, made up according to the manufacturers protocol, for four minutes with occasional gentle agitation. Developing was stopped and excess developer was removed with a ten seconds wash in distilled water with continuous agitation. Fixation was for five minutes in Amfix (1:3 dilution in dH_20) with occasional agitation, and was followed by two wash steps of ten minutes each in distilled water.

Excess film was removed from the back, sides and frosted ends of slides with a razor blade before slides were lightly stained in 1% Methyl Green, allowed to dry thoroughly in air and mounted underneath coverslips in DPX mountant.

Modifications:

Three modifications were found to improve the auto-radiography. 1) A thicker layer of film improved signal to noise ratio, and could be achieved by dipping slides a second time in emulsion a minute or so after first dipping, once the first layer had become firm. 2) The emulsion was less prone to crack if it set before drying, and so after dipping the slides were kept moist rather than being placed with desiccant straight away. 3) Histological information was much improved by a longer staining (30-45 seconds) in Methyl Green.

2.8 Electronic data processing.

2.8.1 Capturing bright and dark field images electronically.

Capturing images was done using a Xillix Microimager 1400 charge coupled device (CCD), camera mounted on a Leitz Axioplan photomicroscope. The camera was controlled by program XMGRAB (MRC custom written) running in X-windows on a Sun workstation. With the shortest available sampling time for this camera of 20 ms, the normal working bright field illumination is too intense, and gives insufficient contrast. To cope with this, the illumination voltage was set as low as possible (3V from a maximum of 12) and a blue filter was applied, giving sampling times 20-50 ms with most Methyl Green stained sections. For lightly stained tissue a second red gelatine filter was added between the sample and the objective to improve the contrast.

The profile of intensity across the desired image was displayed and either the gamma setting of the camera look up table, or the sampling time, was adjusted to give a broad spread of intensity without significantly truncating the distribution.

There is variation in illumination due to the filament of the light source and the optics of the condenser and lenses. The effect of this on the bright field image can be subtracted if an average illumination, in the absence of tissue, can be obtained. Once the sampling time and look up table were set, shading was established by capturing images of blank regions of a slide and using a program that averages several such images including data only if it is brighter than the previous average.

Focusing the images was performed by eye on the computer screen. Variance-based methods exist to assist with this, but did not prove to be advantageous in general. Grabbed images had shading automatically subtracted, and were then saved. A dithered template was made of the first image to ensure that the dark and bright field images were correctly aligned.

To obtain adequate illumination for dark field images, light source voltage was set high (12V) and filters were not generally used. For low power images, the dark field condenser gave rings of dark and light comparable in scale to the size of the image if the conventional condenser was also used. In such cases a more uniform illumination was obtained by using the dark field condenser alone and lowering the condenser stage. For high power images both condensers were necessary. Scanning times were typically 50- 300 ms. As some regions were often exceptionally bright it was sometimes necessary to use camera look up table gamma settings of 0.6-0.8 to capture the range of signal levels without truncating the bright end of the distribution. This procedure used lower levels of illumination, which also helped eliminate the tendency of such bright spots to overload the camera giving smears of maximum intensity. These smears were often due to debris on the slide rather than signal and

gave a clear peak of intensity above that seen for the rest of the tissue. They could thus also be filtered out by thresholding the inverted image at a later stage.

2.8.2 Computer overlaying of signal and histology.

This utilised the program XOVERLAY (MRC custom written). The bright field images were not usually manipulated except to map them into a grey scale of 254 or 252 instead of 255. This left a single bit (plane 1) or two bits (planes 1 and 2) free to carry the signal component of the final image.

The signal was extracted from the dark field image by using only those parts of the image with a brightness above a certain threshold. The dark field image was usually grabbed and immediately thresholded so that the image obtained could be directly compared with what was present under the microscope. Another useful rule-of-thumb is that thresholding should subtract the background illumination, not due to specific hybridisation, and so areas of the section not containing any tissue provide a useful baseline, as much of this background should be removed.

The thresholded dark field image was either mapped directly onto plane 1 or else dithered onto plane 1. Thresholding alone gives no distinction between high and low levels of signal and requires a high threshold to be set. Dithering gives an impression of the different levels of signal, according to how close together shaded pixels are placed, and less information is discarded as lower thresholds are used. This method was thus generally preferred.

The colour of the plane 1 (or of planes 1 and 2 when overlaying two signals) could be set as desired when displaying the images. If the intensity of this plane was not 100%, some impression of the underlying tissue was visible through the coloured

signal. For this colour intensities of 85-94% were used, but with this procedure, it was extremely difficult to later change the colour corresponding to signal, if this was required. Most images captured later in the project thus used a signal colour intensity (weight) of 100%, to allow the colour to be changed.

Overlayed images were displayed as an X-window at full screen size and could be saved using the command XWD which dumps the content of a X-window to a file. Screens were also photographed using Fuji 100ASA daylight slide film using a dark hood. Exposure times were at least 0.25 seconds to eliminate brightness differences from the redisplaying of the screen.

2.8.3 A simpler method for overlays.

A simpler method was used to obtain overlayed images in black and white. Bright and dark field images are captured as normal and converted to a portable anymap (PNM) format using WOOLZTOPNM. Using XV, the colour map of the dark field image was first inverted and then adjusted so that there was a steep gradient of grey scales with signal as black and everything else as white (again using the image down the microscope as reference). The widely available program PNMARITH (Jef Poskanzer) was then be used to multiply together the bright field and dark field images. This procedure treated greys as a scale between black=0 and white=1. As a result the product was the bright field image everywhere except where there was signal. With signal the product was zero so and a black pixel was produced.

This procedure is simple and effective, but the starting bright field image must have no black of its own for an unambiguous final image to be produced. This

method was routinely used to provide scale information as the histology images could be easily merged with a black and white image of a graticule captured at the same magnification.

2.8.4 Composite images.

Separate images were combined into figures and annotated by Norman Davidson using Adobe Photoshop, for which the file format of the X-window output was first changed to a portable anymap (PNM) format using XWDTOPNM, and then to a TIFF format using XV or PNMTOTIF. Care was taken to ensure that these changes in file format did not alter the patterns of signal. Combining images into figures was also performed using PNMCAT which concatenates two or more PNM format files.

2.9 Whole mount *in situ* hybridisation

Digoxigenin labelled riboprobes were made in total volumes of 40µl using DIG RNA labelling kit, (Boehringer Mannheim, Cat no 1175 025) essentially according to Wilkinson (1992), but with the following modifications. A higher concentration of DTT was used (0.025M) and twice the quantities of enzymes were used since additional RNA polymerase(20 units/reaction) and RNase inhibitor(100 units/reaction) were added after one hour of the two hour, 37^{o}C incubation step. For *Pax6* , since a shorter, gene-specific, PCR product was used as the template in place of linearised plasmid, the quantity of DNA used was reduced from 1µg to 300 ng. Embryos were dissected in ice cold sterile phosphate buffered saline (PBS) and fixed overnight in 4% paraformaldehyde in PBS, pH 7.2. To reduce trapping of

unhybridised probe in brain ventricles, heads were dissected away from bodies for older embryos (E11.5) following the initial fixation. Embryos were washed in ice-cold PBT (PBS + 0.1% Tween 20), dehydrated through a methanol:PBT series, stored in 100% methanol at -20 ^{o}C and rehydrated though the methanol:PBT series when required. These steps were performed as described by Wilkinson (1992), except that wash times were increased to 10 minutes per solution. The prehybridisation steps were also based on the protocol of Wilkinson (1992). Washes in PBT and in fresh 2 mg/ml glycine in PBT were increased from 5 to 10 minutes per solution between the bleaching in 6% H_2O_2, digestion with 10mg/ml Proteinase K, and fixation steps. Embryos were equilibrated to the hybridisation buffer by one, 10 minute wash in 1:1 PBT and hybridisation buffer (50% deionised formamide, 5 × SSC, 50 mg/ml heparin, 0.1% Tween-20, pH 5.0), followed by one, 10 minute wash in hybridisation buffer. Pre-hybridisation was for 1-5 hr at 70^{o}C in hybridisation buffer with 100 mg/ml denatured salmon sperm DNA (dssDNA), 100 mg/ml tRNA.

Probes were quantified against DIG labelled RNA of known concentration, by serial dilution and antibody conjugate colour reaction using the Digoxigenin Detection Kit (Boehringer Mannheim, Cat 1210 220) according to the manufacturers protocol. Probe was denatured at 80^{o}C for 5 minutes, cooled on ice and added to hybridisation mix. Hybridisation in 10 ml polystyrene tubes (Sterilin) was at 70^{o}C overnight in a total volume of 1 ml with 50 -100 ng/ml labelled probe, 100 mg/ml dssDNA, 100 mg/ml tRNA. To reduce the risk of desiccation, additional hybridisation mix was placed in a placed in a 0.55 ml microcentrifuge tube at the top of each 10 ml chamber.

Two initial 10 minute posthybridisation washes in prewarmed hybridisation mix at 70°C were followed by a high stringency wash regime of two 5 minute washes and three, 30 minute washes all in 2 × SSC, 50% deionised formamide, 0.1% Tween-20 at 65°C. Embryos were allowed to cool to room temperature and were briefly washed in Tris buffered saline with Tween-20, 1 × TBST (0.025 M Tris/HCl pH 7.5, 8g/l NaCl, 0.2g/l KCl and 0.1% Tween-20). Blocking was by incubating for 1 hour at room temperature in 10% heat inactivated (70°C , 30 minutes) sheep serum in TBST with 0.1% m/v sodium azide.

A 1:2,000 dilution of anti-digoxigenin-alkaline phosphatase in 1% heat inactivated sheep serum, preabsorbed with mouse powder, was produced according to Wilkinson (1992). The 10% heat inactivated sheep serum was replaced by diluted, preabsorbed antibody conjugate for overnight incubation at 4°C, then embryos were washed three times, 5 minutes each in 1 × TBST, then three to five times, 30 minutes-1 hour each, in TBST at room temperature. Embryos were equilibrated with the alkaline phosphatase buffer, (100mM NaCl, 50mM $MgCl_2$, 0.1% Tween-20, 100mM Tris pH 9.5, + levamisole at 1 drop/ 5 ml), by three, 10 minute washes.

For staining, embryos were transferred to glass dishes with 1-2 ml freshly prepared staining solution. 20 mls of staining solution was 0.1M Tris, pH 9.5, 0.05M $MgCl_2$, 0.1M NaCl, containing 90 µl NBT (100 mg nitroblue tetrazolium chloride/1.3 ml 70% dimethylformamide, 30% H_20) and 70 µm X-phosphate (5-bromo-4-chloro-3-indoyl-phosphate, 50 mg/ml in dimethylformamide). Staining was performed in the dark, with embryos being examined every few minutes. Reactions

were stopped by three rinses in 1 × PBT, 1mM EDTA and fixed in phosphate buffered formalin or 4% paraformaldehyde in PBS.

2.10 Scanning electron microscopy.

Scanning electron microscopy was performed with the help of Andrew Ross who processed samples from the post-fixation through to the sputter coating. Samples for electron microscopy were fixed in 2.5% glutaraldehyde. All fixation and wash steps were in 0.1M cacodylate buffer pH 7.3 with 0.1M sucrose, except the dissection which was performed in Phosphate Buffered Saline (PBS) and the osmium post-fixation, performed in 0.1M cacodylate buffer pH 7.3 without additional sucrose. Following overnight fixation embryos were washed then, using sharpened tungsten needles, heads were removed and then bisected along the midline. Half-heads were then fixed again overnight in 2.5% glutaraldehyde, washed twice for ten minutes each, post-fixed in 1% osmium tetroxide, washed briefly and then dehydrated. Dehydration of the samples was using in a graded series of acetone:distilled water with, 25%, 50%, 75% and 100% acetone and three washes, for 5 minutes, 10 minutes and 15 minutes respectively, at each acetone concentration. Critical point drying was performed in a Balzers CPD020 using CO_2 as the transitional fluid. Dried samples were mounted onto stubs using Devcon epoxy resin and sputter coated with platinum using Polaron E5100. Samples were viewed on a Hitachi S800 field emission scanning electron microscope at 5 kV, and photographed using Kodak T-MAX 400 film.

2.11 Transmission electron microscopy.

Transmission electron microscopy was performed with the help of Andrew Ross, who processed the fixed dissected samples through to Araldite blocks, cut the thin sections, and provided training in the use of the microscope.

Samples for transmission electron microscopy were fixed overnight in 2.5% glutaraldehyde. All fixation and wash steps were in 0.1M cacodylate buffer pH 7.3 with 0.1M sucrose, except the dissection which was performed in phosphate buffered saline (PBS) and the osmium post-fixation, performed in 0.1M cacodylate buffer pH 7.3 without additional sucrose. Fixed heads were removed and then bisected along the midline with sharpened tungsten needles. Half heads were fixed again in 2.5% glutaraldehyde for at least 2 hours then rinsed twice. Postfixation was for 1 hour in 1% osmium tetroxide. Samples were rinsed and then dehydrated through an ethanol:distilled water series (10% ethanol, 2 × 5 minutes, 1 × 10 minutes; 20%, 30%, 50%, 70%, 95% ethanol , 10 minutes each; 100% ethanol 3 × 10 minutes). Samples were transferred to propylene oxide (2 × 10 minutes) and then infiltrated overnight in Araldite. The Araldite was prepared by mixing 10 ml Araldite resin CY 212, 10 ml DDSA and 0.5 ml Dibutyl phthalate for 1 hour, then adding 0.5 ml DMP 30 accelerator and mixing for a further hour before adding samples. The Araldite with samples were heated to 60°C for 35 minutes then samples were transferred into embedding Araldite and kept at 60°C for three days to harden.

Semithin (1μ) sections were cut on a LKB 2088 Ultrotome V, heated onto slides and stained with 1% Toluidine Blue 1% borax to determine the position at

which thin sections should be cut. Thin sections were cut at a thickness of 50 nm, floated onto grids and viewed on a Philips CM 10 transmission electron microscope.

2.12 Detection of cell death with Nile Blue Sulphate

Cell death was detected using Nile Blue Sulphate staining (Sulik *et al.,* 1988). Freshly dissected embryos were stained in 1:20,000 Nile Blue Sulphate in either PBS or PBT (PBS with 0.01% Tween 20) for 15 minutes and photographed immediately using 64T film on a Wild M400 photomicroscope. The specificity of this procedure was confirmed by comparing the pattern of dark-staining cells with that generated using Acridine Orange DNA stain, (Graham e*t al.*, 1993).

2.13 *Msx1* transgenic mice

The line of *Msx1/lacZ* transgenic mice used was ΔH6 and was provided by Bob Hill. The reporter construct contains 5 kb of sequence upstream of the mouse *Msx1* gene and generates a fusion transcript with *lacZ* in frame with the *Msx1* gene from an *Nco* I site 127 bp 3' of the start of the *Msx1* coding region. The construct uses SV40 poly(A) addition and transcription termination sequences. Transgenic mice were produced on a (CBA X C57BL/6)F_1 hybrid background. From matings of Swiss *Sey* mice to transgenics, *Sey* males carrying the transgene were identified by Southern hybridisation and PCR analysis. These were mated to *Sey* females. Embryos from these matings were obtained at E9.0 to E11.5 days and fixed in 2% formaldehyde, 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3), 2 mM $MgCl_2$, 5 mM EGTA, then stained for β-galactosidase activity. Fixation times were 10-30 minutes depending upon embryo size. The staining procedure was essentially

that published for use in lineage marking studies (Protocol 9 in Beddington and Lawson, 1990). Fixed embryos were washed three times in a detergent wash, (0.1M phosphate buffer, pH 7.3 with 2mM $MgCl_2$, 0.1% (w/v) sodium desoxycholate, 0.02% (v/v) Nonidet NP40 and 0.05% bovine serum albumin (BSA)) and transferred to staining solution. Staining solution was based on the detergent wash without BSA. It contained 5mM ($K_4Fe(CN)_6.6H_20$), 5mM($K_3Fe(CN)_6$), 0.001% NaCl and 1mg/ml of 5-bromo-4-chloro-3 indol-β-D-galactopyranoside (X-gal) diluted from a 50 mg/ml stock in dimethyl formamide. Staining solution was filtered through Whatman's Nº. 1 paper before use. Staining was for 5 hours in the dark at 37°C. Embryos were rinsed two or three times in detergent wash and postfixed in 4% paraformaldehyde or in phosphate buffered formalin.

2.14 Photomicroscopy of sections and embryos.

Fixed embryos were dissected if required using tungsten needles and sharp forceps and photographed on Wild M400 or Leitz Ortholux microscopes using Fuji 64T film (colour transparencies) or Technical Pan (black and white print) film. If complex positioning of embryos was required, they were propped in indentations cut into a plate of 1% agarose (made up in PBS), on the bottom of a petri dish, and viewed with incident, as well as transmitted, light. On the M400 microscope, the Fuji 64T film was rated at ISO/ASA 64 for bright-field illumination, and at ISO/ASA 200 for dark-field illumination with exposure times lengthened from the exposure meter readings by a factors of 1-1.41 and of 1.41- 2 respectively. Technical Pan was rated at ISO/ASA 50 and ISO/ASA 200 for dark and bright field respectively, with exposures

given by the meter reading or increased by a half or one *f* stop (1.41-2). Using the Ortholux microscope, both types of film were rated as ISO/ASA 25 for bright field images and ISO/ASA 50 for dark field images, and the exposures was set automatically from an estimate of the sample to whole frame area.

CHAPTER 3

Results from studies addressing the roles of the *Pax6* gene in eye and nasal development.

Description of the experiments in this chapter.

The aim of the work presented in this chapter was to identify the roles of the *Pax6* gene in eye and nasal development. Related work, aimed at identifying roles for *Pax6* in brain development and the basis of early forebrain defects of *Sey/Sey* embryos, is presented separately in Chapter 5, and discussed in Chapter 6.

As stated at the end of the introduction, information on possible roles for the *Pax6* gene can be obtained either by examining the *Small eye* homozygous mutant phenotype or by considering the expression patterns of *Pax6* mRNA in wild-type animals. The approach taken has been to obtain both types of information and then combine the information directly by analysing the mutant phenotype with molecular markers, including the expression of *Pax6* itself. This chapter is divided into four sections following this format. The first section describes the defects in eye development in homozygous *Small eye* animals. The second section is an analysis of the normal pattern of *Pax6* mRNA expression during eye development. In the third section, the eye phenotypes described in the first section are analysed using molecular markers, in particular the expression of the *Pax6* gene. The final section describes the results obtained for the analysis of *Pax6* roles in nasal development, with expression data and phenotypic descriptions presented together.

3.1 Abnormalities of eye development in *Sey/Sey* embryos.

3.1.1 An early optic vesicle shape abnormality.

Around E8.5, normal optic vesicles are considerably broader proximally, at their base, than they are distally (see Fig. 3.11 B). Producing an optic cup and stalk requires a relative expansion of the distal region and constriction of the proximal optic vesicle (Kaufman, 1979). Normally this process is well advanced by E10.5 when the optic vesicle has formed a bulbous, bi-layered optic cup and narrower optic stalk. Examination of transverse sections of eyes at E10.5-E11.5, revealed that *Sey/Sey* mutants typically had optic vesicles that were broader than normal, but distorted at their distal ends (shown at E11.5 in Fig. 3.2, F). These optic vesicles were of uniform width along most of their length, as though the proximal restriction of the optic vesicle had not occurred. Examination of whole mount preparations and sections at E9.5 identified a class of embryos which both had this broad proximal optic stalk and had a failure to form furrows on the surface of the prosencephalon. A genotype analysis of these embryos was performed by Bob Hill, using polymerase chain reaction (PCR) and *Dde*I digestion. This confirmed that the class of embryos, morphologically identified on the basis of optic vesicle and brain shape, were homozygous *Small eye* mutants (Fig. 3.1).

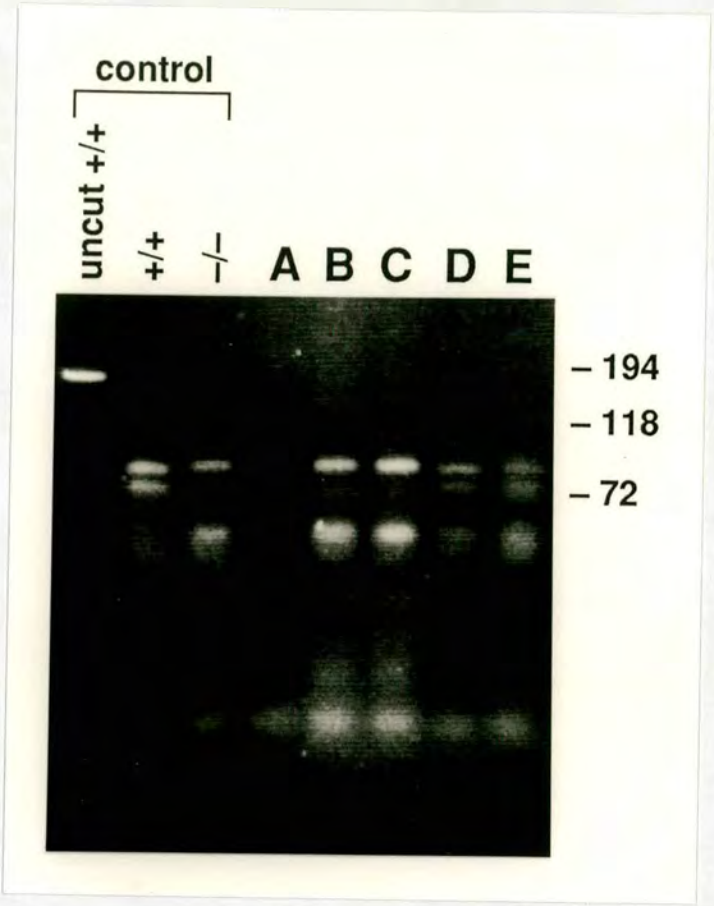

Figure. 3.1

PCR genotyping of young embryos. Proteinase K treated embryo biopsies have been used as templates for PCR amplification of a fragment of the *Pax6* gene containing the site of the *Sey* mutation. The *Sey* mutation creates an diagnostic *Dde*I cleavage site, thus genotypes of embryos are determined by running *Dde*I digestion products on a 4% NuSieve gel. The wild-type amplified fragment (lane one) contains a second *Dde*I site as a positive control for digestion, thus giving bands of 83 bp and 71 bp when digested (lane two). In the *Sey* mutation, the fragment in this lower band is cut to give 51 bp and 20 bp fragments (see lane three). A-E are biopsies from five embryos aged E8.0 and E8.5. Two of the embyos (B and C), are shown by the assay to be homozygous for the *Small eye* mutation. Embryos D and E are both *Sey*/+ , and the sample from A has failed to amplify. Positions of molecular size marker bands are indicated on right hand margin. The *Sey/Sey* (-/-) control used in this case is a sample from the embryo shown in Figure 3.2 B, whose genotype had been determined in a previous experiment.

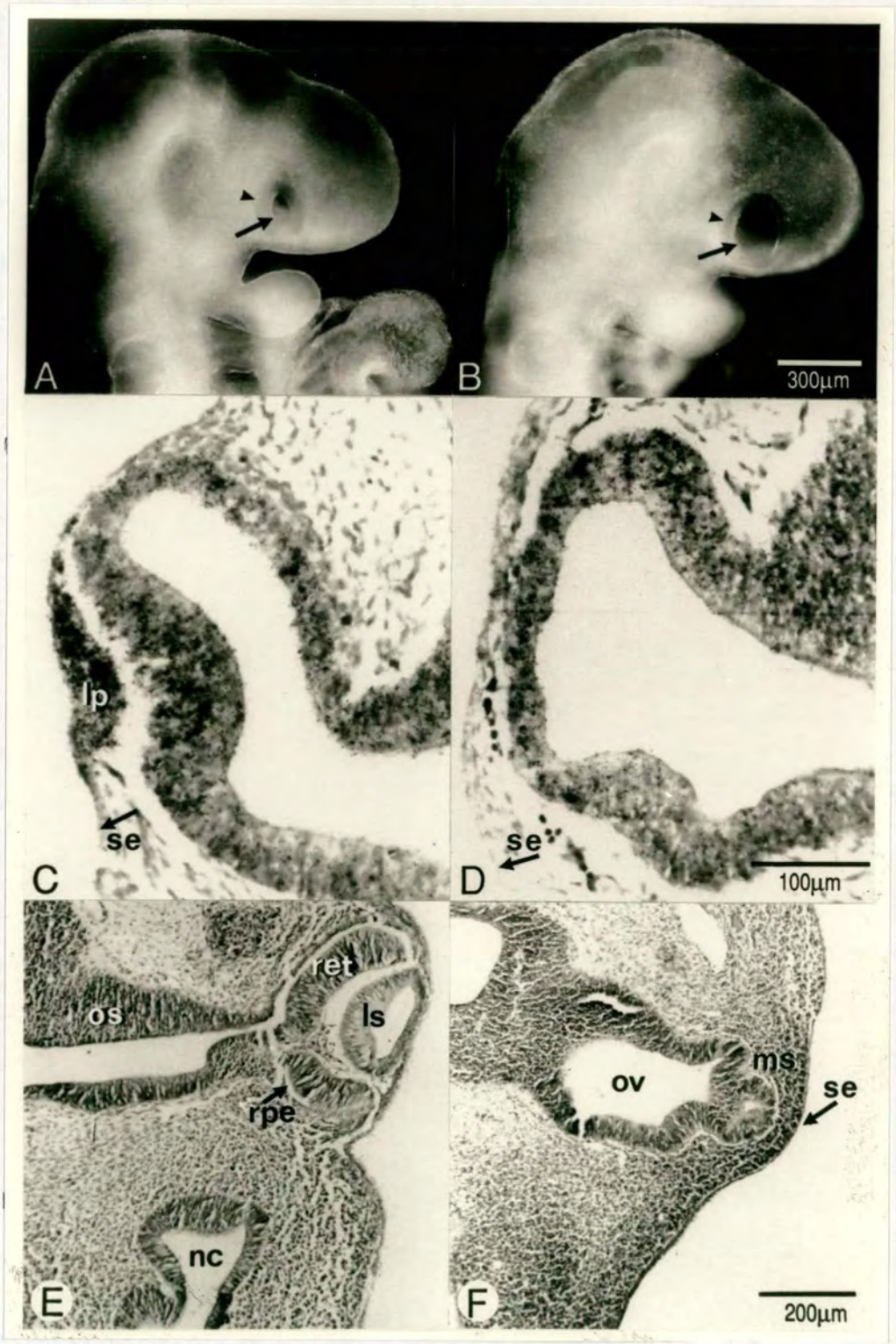

Figure. 3.2 (see following page for legend)

3.1.2 Distal distortions of *Sey/Sey* optic vesicles.

In normal eye development the optic vesicle interacts with the lens pit, growing around it to form a bi-layered optic cup. The thin outer layer and thicker inner layer of the optic cup go on to form pigmented retinal epithelium and neural retina respectively. The distorted distal ends of *Sey/Sey* optic stalks (Fig 3.2 F) often give the appearance of an abortive process of optic cup formation (Fig. 3.12 A-C, E, G). Optic vesicles in E11.5-E15.5 S*ey/Sey* embryos display a bi-layered neuroepithelial structure. The *Sey/Sey* optic stalk retains a lumen (Fig. 3.12), unlike the normal optic nerve, which is a dense bundle of axons by E15.5.

Figure 3.2 (preceding page)

Early eye phenotype of *Sey/Sey* embryos (right column) and controls (left column). Characteristic appearance of the eye at E9.5 of a wild-type embryo (A)and *Sey/Sey* embryo (B). *Sey/Sey* optic vesicle is broader than normal and has failed to constrict proximally. Arrows, proximal restriction of optic vesicles. Arrowheads, extent of the eye region.

Histology of the eye at E9.75 (C),(D), and E11.5 (E),(F). At E9.75, the lens placode (lp), a prominent thickening of the surface ectoderm (se) is present in the littermate (C), but absent from the *Sey/Sey* embryo (D). Later *Sey/Sey* embryo, at E11.5 in (F) lacks the developing lens (ls), and nasal cavity (nc), present in littermate (E). Normal littermate optic vesicle has produced an optic stalk (os), and optic cup with distinct retina (ret), and pigmented retinal epithelium (rpe). *Sey/Sey* optic vesicle (ov), is broader than normal, is distorted at the distal end and is separated from surface ectoderm (se) by intervening mesenchymal-like cells (ms).

3.1.3 Absence of lenses in *Sey/Sey* embryos.

Sey/Sey embryos from E 10.5 to E15.5 were examined by haemotoxylin and eosin histology for evidence of lens formation. For stages E12.5 onwards, when lens fibre differentiation could be detected in controls, there was no evidence of lenses, lentoid bodies or of cells with the eosin staining properties of lens fibres in any homozygous mutant examined. Thus it appears that a lens neither forms and then degenerates, nor forms much later in development, but rather is never present.

Normal early lens development involves the thickening of surface ectoderm to form a lens placode, and its subsequent invagination to form a lens pit and lens vesicle. Two features of *Sey/Sey* embryos could be confused with these stages. A dense mass of tissue is observed in contact with the distal optic vesicle, but this appears to be condensed mesenchyme rather than thickened ectoderm (see Fig. 3.12 E, G). Secondly, a pit is observed in the skin of later *Sey/Sey* animals, but reflects eyelid formation. These two features will be described later (in sections 3.1.8 and 3.1.9 respectively). With these observations eliminated there is no evidence from the histological analysis for any formation of thickened surface ectoderm in the eye region.

3.1.4 Absence of lens placodes in *Sey/Sey* animals.

Characteristic optic vesicle shape and brain shape abnormalities allowed *Sey/Sey* embryos to be recognised earlier than was previously possible (section 3.1.1), hence *Sey/Sey* mice could be identified during the first morphologically recognisable stage of lens development, the formation of an lens placode from surface ectoderm.

The lens placode is normally forming at E9.5 in the mouse and is fully present by 9.75, when it is readily detected by light microscopy (Fig 3.2, C). Histological analysis of *Sey/Sey* embryos at this stage revealed no evidence of lens placode formation (Fig. 3.2 D).

In later histological sections the *Sey/Sey* optic vesicle can be seen to be separated from the surface ectoderm by intervening mesenchymal cells (shown at E11.5 in Fig. 3.2 F). The absence of lens placode and the disruption of the surface ectoderm interaction with the optic vesicle were studied by transmission electron microscopy (TEM).

Figure 3.3 (following page)

Morphology of eye region surface ectoderm. Transmission electron microscopy (TEM) of control (left column) and *Sey/Sey* (right column) surface ectoderm around the eye at E9.75. (A) Away from the eye region, surface ectoderm (left) has a cuboidal morphology. (B) Adjacent to the optic vesicle (top right) the surface ectoderm forms a lens placode, with narrow elongated cells. (C) Towards the thickest part of the lens placode there is extreme elongation of surface ectoderm cells. Nuclei are positioned basally, and there are numerous, apically-positioned mitochondria. (D) In the *Sey/Sey* mutant, surface ectoderm away from the eye region is comparable to that in controls (A). (E and F) Adjacent to the optic vesicle , there is some thickening of surface ectoderm, but the relative elongation of cells seen in (C) is not found. Neural ectoderm of the optic vesicle (ne) and surface ectoderm (se) are largely separated by inervening mesenchymal cells (mes).

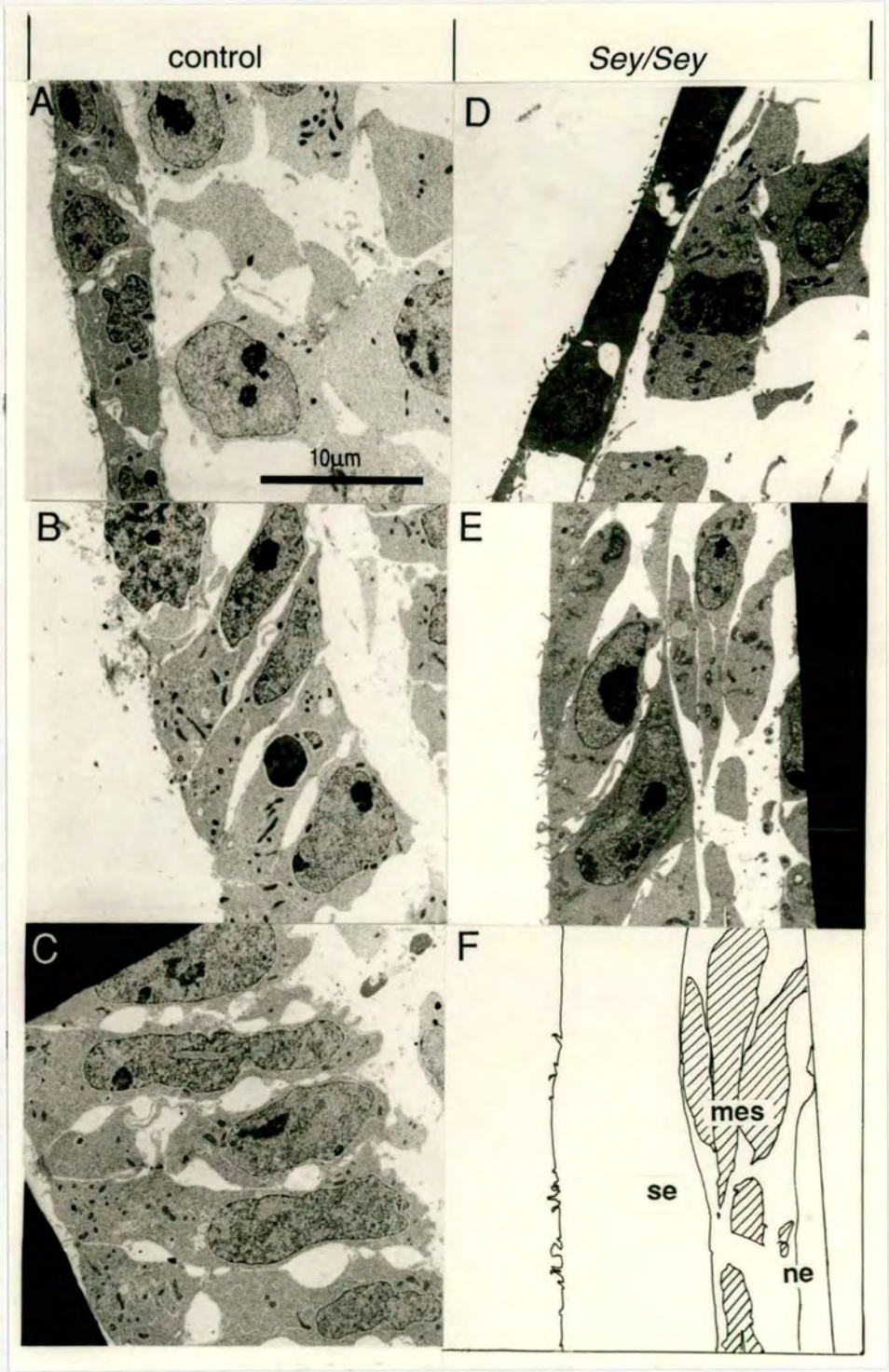

Figure 3.3 (see preceding page for legend)

Figure 3.3 shows the results of TEM analysis of the eye region between E9.75 and E10 based on horizontal and frontal sections from *Sey/Sey* embryos, D,E or their littermates (*Sey*/+ and +/+), A-C. Away from the eye (laterally and dorsally), surface ectoderm cells in control embryos typically have a cuboidal shape (Fig. 3.3 A). In the lens placode, adjacent to the optic vesicle, the surface ectoderm is made up of narrower, more elongated, cells with widely separated apical and basal surfaces (Fig. 3.3 B). Not all of the most prominent regions of lens placode are directly in contact with the optic vesicle at these stages however. Figure 3.3 C shows a more fully developed region of the placode, which is underlain by mesenchymal cells. The surface ectoderm cells here are even more elongated, and typically have a basally located nucleus and a clustering of mitochondria towards the apical surface.

In homozygous mutant embryos, the surface ectoderm away from the eye region was similar to that in controls (Fig. 3.3 D). Near the optic vesicle there was some thickening of the surface ectoderm (Fig. 3.3 E), which was typically separated from the neural ectoderm by intervening mesenchymal cells (Fig. 3.3 F). At the stages analysed, *Sey/Sey* surface ectoderm cells never achieved the placodal morphology shown in Fig. 3.3 C.

Some *Sey/Sey* surface ectoderm cells thus achieve an apparently intermediate form between cuboidal and a true placode morphology. The significance of this is questionable, however. In normal embryos there is variation in surface ectoderm thickness over the head. In particular, there is a ridge of thickened surface ectoderm that runs in an anterior to posterior direction, ventrolaterally below the eye region. At the posterior of this ridge there complex ectodermal thickenings of the future

maxillary process, the periphery of Rathke's pouch and the groove between the optic eminence and the first branchial arch (the proximity of the jaw to the eye at these stages can be seen in Fig. 3.2 A and B). In frontal sections away from the eye and nasal regions, surface ectoderm is thin dorsally, becomes thicker laterally, forms a greatly thickened ridge ventrolaterally, and becomes thinner again at the ventral midline. Where a placode is present however this dorsal to ventral profile involves a considerable thickening of surface ectoderm, then a thinning ventral to the placode, followed by a thickening again to form the ridge, which has a looser arrangement of cells that the placode itself. On frontal sections of *Sey/Sey* eyes, no convincing example of surface ectoderm thinning was found dorsal to the ventrolateral ridge. Thus the observed thickening in the eye region may be in part be related, not to placode formation, but to the underlying dorso-ventral variation surface ectoderm thickness.

3.1.5 Occasional close proximity of surface ectoderm and optic vesicle.

Although the surface ectoderm is mainly separated from the optic vesicle by intervening mesenchymal cells in *Sey/Sey* mutants, many sections show regions where there are no such cells. An example is shown in Fig 3.4. A composite of neighbouring images for this example (Fig. 3.5) shows that the outer profiles of both surface ectoderm and optic vesicle are relatively smooth, and that the inclusion or exclusion of mesenchymal cells in this region is accommodated by variation in surface ectoderm thickness. Outlines of individual surface ectoderm cells (shaded) suggest that they may be more elongated when in close proximity to neural ectoderm and more cuboidal if separated from the optic vesicle by mesenchymal cells.

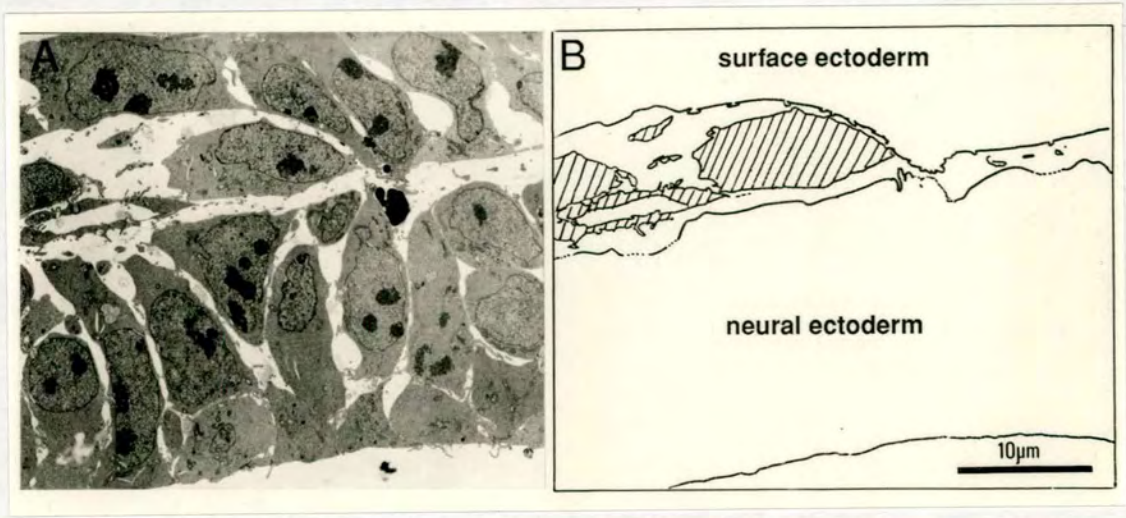

Figure 3.4

Regions of local contact between *Sey/Sey* optic vesicle and surface ectoderm. TEM section at E9.75 from the eye region of a *Sey/Sey* embryo, with schematic (right) to show cell layers, identified by basement membranes. In the right hand third of the photograph, surface ectoderm and neural ectoderm are in close proximity with no intervening mesenchymal cells.

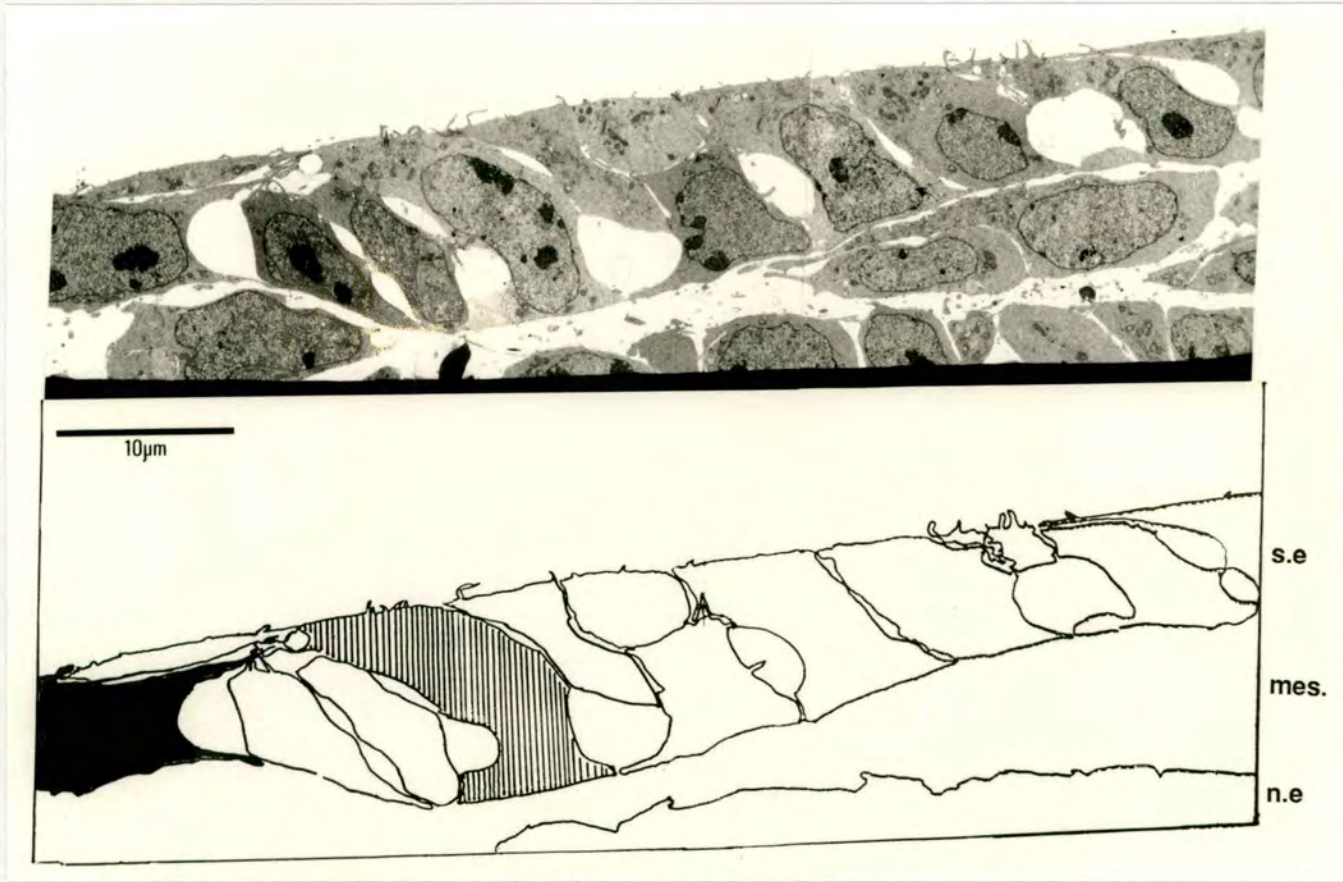

Figure 3.5

Variation in surface ectoderm thickness in *Sey/Sey* eyes. Composite TEM image of several adjacent sections from the eye of a *Sey/Sey* embryo at E9.75. Profiles of the surface ectoderm, mesenchyme and neural ectoderm are shown in the schematic below the photographs. The surface ectoderm thickness varies with the presence or absence of underlying mesenchymal cells. Individual cell outlines (shaded) suggest cell morpology may be more elongated, and less cuboidal, in regions where surface ectoderm and neural ectoderm come into contact.

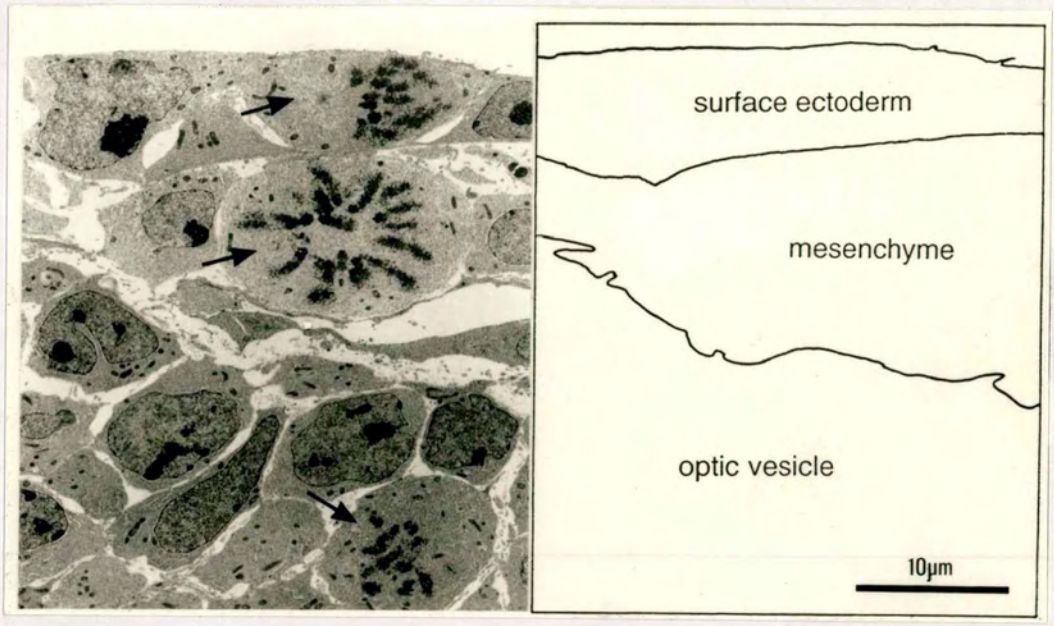

Figure 3.6

Cell division in all three tissue layers in the *Sey/Sey* eye. Transverse TEM section from the eye of a *Sey/Sey* embryo at E10,with schematic (right) showing tissue layers identified by the position of basement membranes. Arrows identify cells with condensed chromosomes that are in mitosis. These are present in surface ectoderm, the optic vesicle and in intervening mesenchyme.

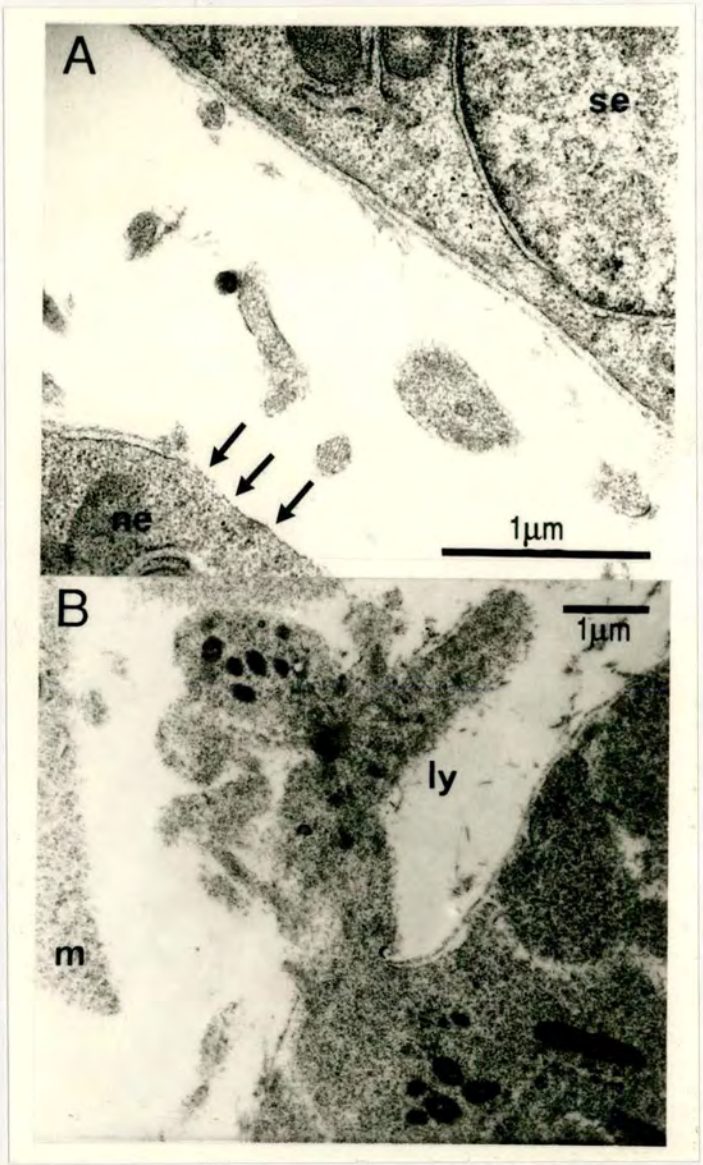

Figure 3.7

Abnormalites of optic vesicle basement membranes, and cell lysis in *Sey/Sey* embryos. (A) In *Sey/Sey* mutants there are extensive breaks (arrows) in the basement membrane of the neural ectoderm (ne) forming the optic vesicle. There are no such breaks in the basement membrane of the seurface ectoderm (se). (B) Lysis (ly) of a neural ectoderm cell from a *Sey/Sey* optic vesicle, through a break in the basement membrane. (m) mesenchymal cell.

3.1.6 Cell division and early eye defects.

Aiming to identify processes affected in *Sey/Sey* eyes, patterns of cell proliferation were examined in TEM sections. No general failure of proliferation occurs at these stages. Figure 3.6 shows that mitotic figures are present in *Sey/Sey* mutant eyes in the optic vesicle, in the surface ectoderm and in the intervening mesenchyme. Division in the layer of intervening mesenchymal cells suggests that this cell population can enlarge both by proliferation as well as by cells migrating in. Thus the division of intervening mesenchymal cells could be important in the increasing separation between surface ectoderm and optic vesicle.

3.1.7 *Sey/Sey* optic vesicles and cell death.

The basement membranes of surface ectoderm and neural ectoderm were used to distinguish the cell layers in TEM sections. In *Sey/Sey* embryos the basement membrane of the neural ectoderm, but not of the surface ectoderm, was frequently disrupted. This occurs in normal eye development (Svoboda and O'Shea, 1987), thus such gaps were also present in control embryos, but appeared to be less frequent and less extensive. An example of this phenomenon from a *Sey/Sey* embryo is shown in Fig. 3.7 A. The example is from a region where surface ectoderm and neural ectoderm are closely apposed, but the basement membrane breaks also occurs where there are intervening cells. Such breaks were not however apparent in the neural ectoderm outside the eye, posterior to the optic vesicle.

One phenomenon observed in *Sey/Sey* optic vesicles but not in controls is shown in Fig. 3.7 B, which shows the lysis of a neural ectoderm cell, spilling its

cytoplasm into the intercellular spaces through a gap in the basement membrane. As well as such necrotic cells, numerous apoptotic cells were present in *Sey/Sey* optic vesicles. These are however common in normal optic vesicles, and their numbers and locations vary with the plane of section. Because of this, the series of sections studied was judged to be insufficiently complete to make any quantitative comparison of cell death.

3.1.8 Condensations of mesenchymal cells.

A region of dense tissue near the surface adjacent to the distal optic vesicle is typically present in homozygous *Small eye* mutants from E10 onwards. This is not abortive or delayed lens development, but is probably a condensation of mesenchymal cells. Developing lens normally strongly expresses mRNA for the extracellular matrix protein gene *entactin* from the lens pit stage onwards (Fig. 3.8 A; Dong and Chung, 1991). In contrast, the regions of dense tissue in *Sey/Sey* embryos express *entactin* mRNA at low levels (Fig. 3.8 B) more comparable to the level of expression in mesenchymal cells that condense adjacent to the anterior optic cup in normal embryos.

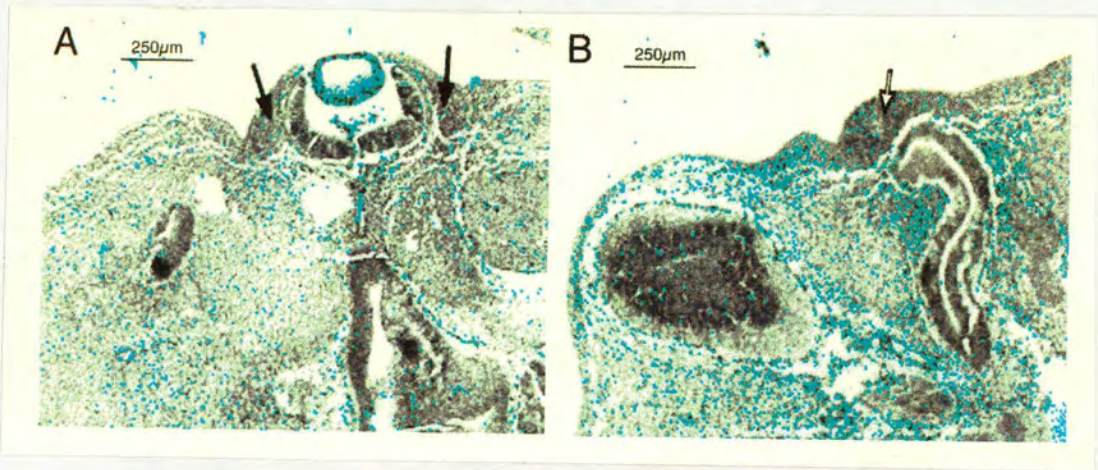

Figure 3.8

Condensations of mesenchymal cells and *entactin* expression. Radioactive *in-situ* hybridisation detecting expression of *entactin* mRNA, blue, in transverse sections at E12 of *Sey/Sey* embryo (B) and normal littermate (A). Developing lens expresses high levels of *entactin* mRNA from E10 onwards, whereas condensing mesenchymal cells, (arrows) around the optic cup in (A) have low levels of expression. A region of dense tissue (open arrow) present at the surface adjacent to the distal optic vesicle in (B) is typical for *Sey/Sey* embryos, and has the appearance and expression characteristics of condensed mesenchyme rather than of developing lens. Anterior of sections is to the left.

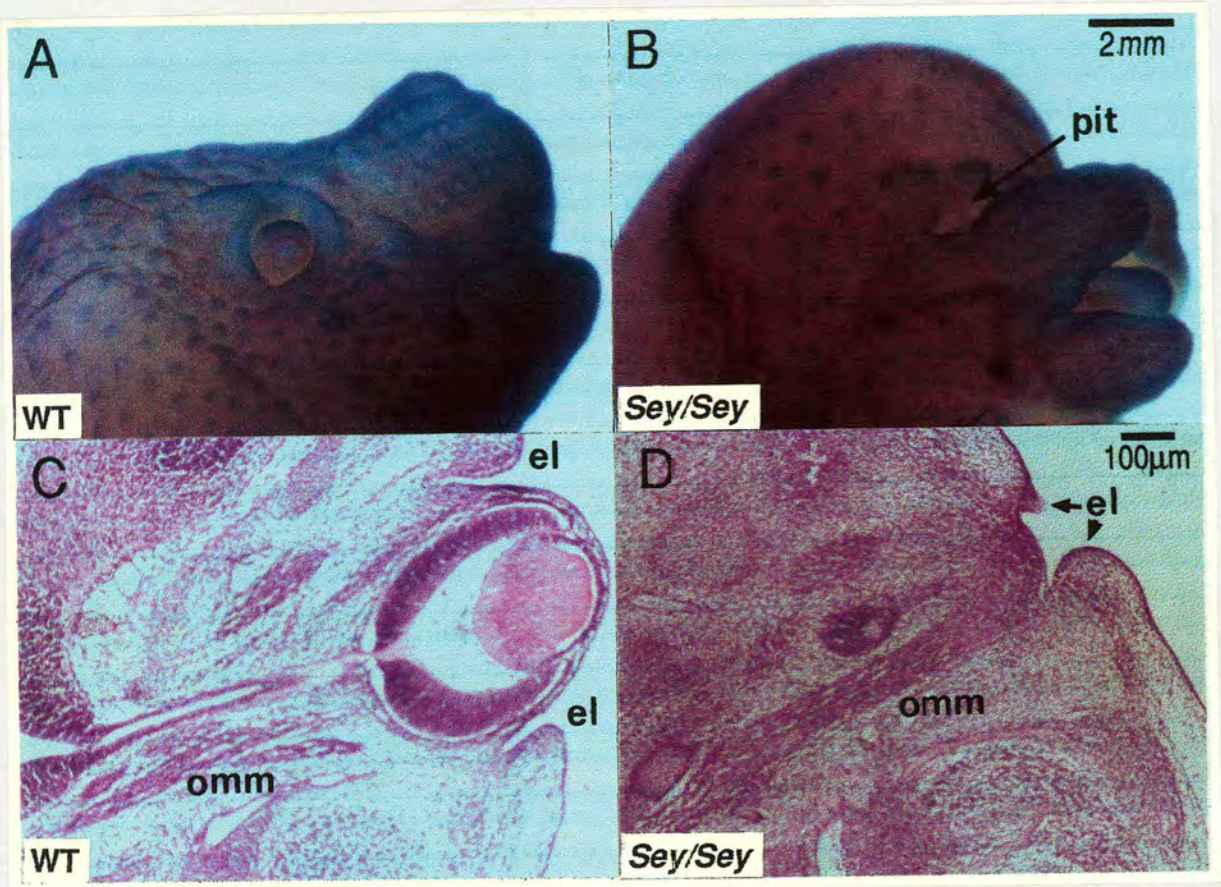

Figure3.9

Eyelids and ocular muscles in *Small eye* homozygous mutant embryos.(A, B) Heads of wild-type embryo (A) and *Sey/Sey* embryo (B) at E14.5, stained with Toluidine Blue to reveal surface morphology. There is a pit in the eye region of the *Sey/Sey* mutant embryo (arrowed in B). (C, D) Haemotoxylin and eosin histology of frontal sections at E13.5 of wild-type embryo (C) and *Sey/Sey* (D)embryos. Ocular muscles (omm), form in *Sey/Sey* embryo, but terminate beneath the pit. Morphology of the structures at the margins of the pit (arrowed) suggests they are eyelids (el).

3.1.9 Eyelids and ocular muscles in *Small eye* homozygous mutant embryos.

During this analysis, one consistent finding in older embryos was the presence of a pit in the skin of homozygous mutants. This was investigated further. The initial studies of eye development in *Small eye* homozygous mutants by haemotoxylin and eosin histological analysis used both Sey^{Neu}/Sey^{Neu} and *Sey/Sey* embryos at E14.5. For both alleles there was a pit present in the skin of the animals. This was approximately where an eye would be expected, but somewhat more frontally placed. This pit was rendered more visible by briefly staining the surface of whole heads in haemotoxylin or in a dilute solution of Toluidine Blue in 70% alcohol. Typical, E14.5, wild-type and *Sey/Sey* embryos stained with Toluidine blue are shown in Fig. 3.9 A,B. The depth and height of these pits were similar in both mutant alleles, but those of Sey^{Neu}/Sey^{Neu} embryos were longer along the antero-posterior axis, producing an elliptical slit rather than a singular pit.

From their appearance, a number of explanations for the origin of these pits could be advanced, including the possibility that they represented lens formation that had reached the stage of producing a lens pit. To aid their identification, the formation of pit structures in *Sey/Sey* animals was related to events in normal eye development by histological analysis, and by scanning electron microscopy (SEM)of the surface of heads. In normal embryos, clefts become apparent around the eye between the eyeball and developing eyelids. These clefts are the future conjunctival sac, which is formed upon eyelid closure. Histologically the appearance of pits at E13.5 and E14.5 is similar to the appearance of the future conjunctival sac, and the structures above and below the pit in mutants (Fig. 3.9 D) resemble eyelids (Fig. 3.9

C). SEM analysis from E11.5 to E16.5 revealed that the pit closed at around E15.5 (Fig. 3.10 B, D), the same time that eyelid fusion occurred in control (*Sey*/+ and +/+) animals (Fig 3.10 A, C) Moreover normal eyelid development at this stage features migratory cells moving into the region and masses of peridermal cells around the edge of the eyelids (Fig. 3.10 C; Findlater *et al.*, 1993). Around the pit in the skin of the *Sey/Sey* mutant skin there are streams of migratory cells (right hand side of Fig. 3.10 D, top of Fig. 3.10F) and threads of peridermal cells (Fig. 3.10 F).

The ocular muscles are produced on schedule in *Sey/Sey* animals and their position initially appears normal. There is no sclera for them to attach to however, and some ocular motor muscles terminate subjacent to the surface pit (Figure 3.9, D). Attachment of these muscles is unlikely to be causal in pit formation however, as an indentation is present in the eye region before the distal ocular motor muscles have formed. Instead it seems likely that the pits observed reflect eyelid formation proceeding in the absence of an eyeball.

Figure 3.10 (see following page)

Scanning electron microscopy (SEM) analysis of the eye region in *Sey/Sey* animals. SEM of heads of *Sey/Sey* embryos (right column) and controls (left column) at E15.5. (A) and (B) are low magnification views of the eye and snout region. The eye is positioned in the towards the top left of the picture in both cases. (C) Higher magnification view of the control eye. The eyelids are nearly completely closed. Streams of long, elonated cells are present migrating towards the eye area, and there is considerable cellular activity involving peridermal cells around the rim of the closing eyelids. (D) Around the pit in the skin of the *Sey/Sey* embryo, migrating cells are present (centre right) and there are clusters of cells within the pit (centrer left), with the morphology of peridermal cells. (E) Periderm cells (per) at the edge of the eyelid region in the control mouse. (F) Cells with a similar morphology to (E) from the pit in skin of the *Sey/Sey* eye region.

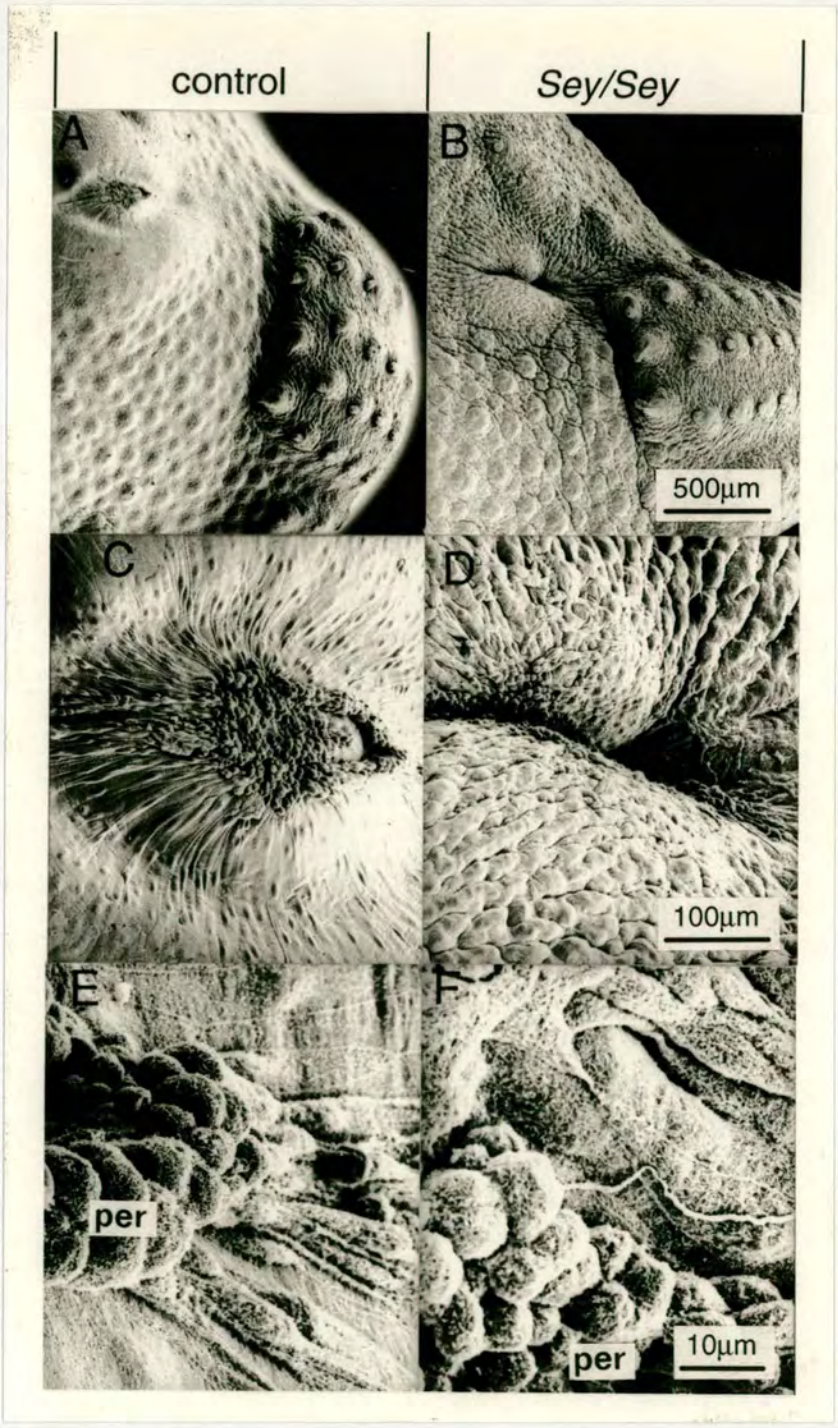

Figure 3.10 (see preceding page for legend)

3.2 Expression of *Pax6* mRNA during eye development in wild-type mice

3.2.1 *Pax6* expression in the neural ectoderm.

I have examined *Pax6* transcription in wild-type mice from 8 days to 15.5 days. At the earliest stage examined, E8.0, *Pax6* transcripts were detected over an extensive region of head neural ectoderm, including the optic pit, the first morphologically detectable indication of the eye region (Fig. 3.11 A). There was no expression in the floor plate or ventral midline. At E8.5, shortly before head fold closure, much of the complexity of the later forebrain *Pax6* expression pattern has been established. In the presumptive diencephalon, expression is mainly dorsal, reaching the neural folds, and is already segmented as has been reported for later stages, (Walther and Gruss, 1991; Figdor and Stern, 1993; Puelles and Rubenstein, 1993). Transverse sections through the head at this stage typically show expression to be strong in the optic vesicle, weaker more rostrally in the presumptive telencephalon, with the neural ectoderm expression being undetectable or only weakly detectable at the neural folds (Fig. 3.11 B).

Pax6 expression within the optic vesicle is polarised distally. Thus, from E9.5 onwards expression in the optic vesicle is strongest around the rim of the developing optic cup (Fig 3.11 D) and is consistently weaker both in the back of the optic cup and in the proximal optic vesicle structures such as the developing optic stalk (Fig. 3.11 E). In the early optic cup, *Pax6* is expressed in both the prospective retinal pigmented epithelium (RPE) layer and prospective neural retina (Fig. 3.11 E). This

pattern is dynamic. Thus, by E15.5 expression within the RPE is only seen in anterior regions, i.e. near the rim of the optic cup. (Fig. 3.11 F).

3.2.2 Restriction of *Pax6* mRNA expression in surface ectoderm of wild-type mice.

At E8.0 *Pax6* mRNA is expressed in a broad region of head surface ectoderm covering the prosencephalon but not the hindbrain region or the presumptive first branchial arch (Fig. 3.11 A). This expression in the surface ectoderm is still broad at E8.5 and extends rostrally to the neural folds. This is in contrast to the neural ectoderm expression at this stage, where strong expression is localised to the optic vesicle and presumptive diencephalon (Fig. 3.11 B). Over the next 24 hours, expression in the surface ectoderm becomes restricted to the developing lens placode (Fig. 3.11 D) nasal placode and immediately adjacent tissues. *Pax6* mRNA expression in the lens placode forms part of a larger domain that extends in the dorsal-caudal direction (Fig. 3.11 C). As lens pit formation proceeds, this expression domain is further restricted until it lies exclusively within the developing eye region (Fig. 3.11 E). Similarly the expression in the ectoderm between the lens and nasal placodes persists longer than most non-placodal expression, but by E9.75 the lens and nasal placodes are clearly separated by a region of non-expressing ectoderm. Consistent with a previous report (Walther and Gruss 1991), *Pax6* mRNA expression was found to continue in the parts of the eye derived from surface ectoderm until the last stage examined, E15.5 (Fig. 3.11 F). This includes the lens pit, the lens vesicle and the lens as well as the developing cornea.

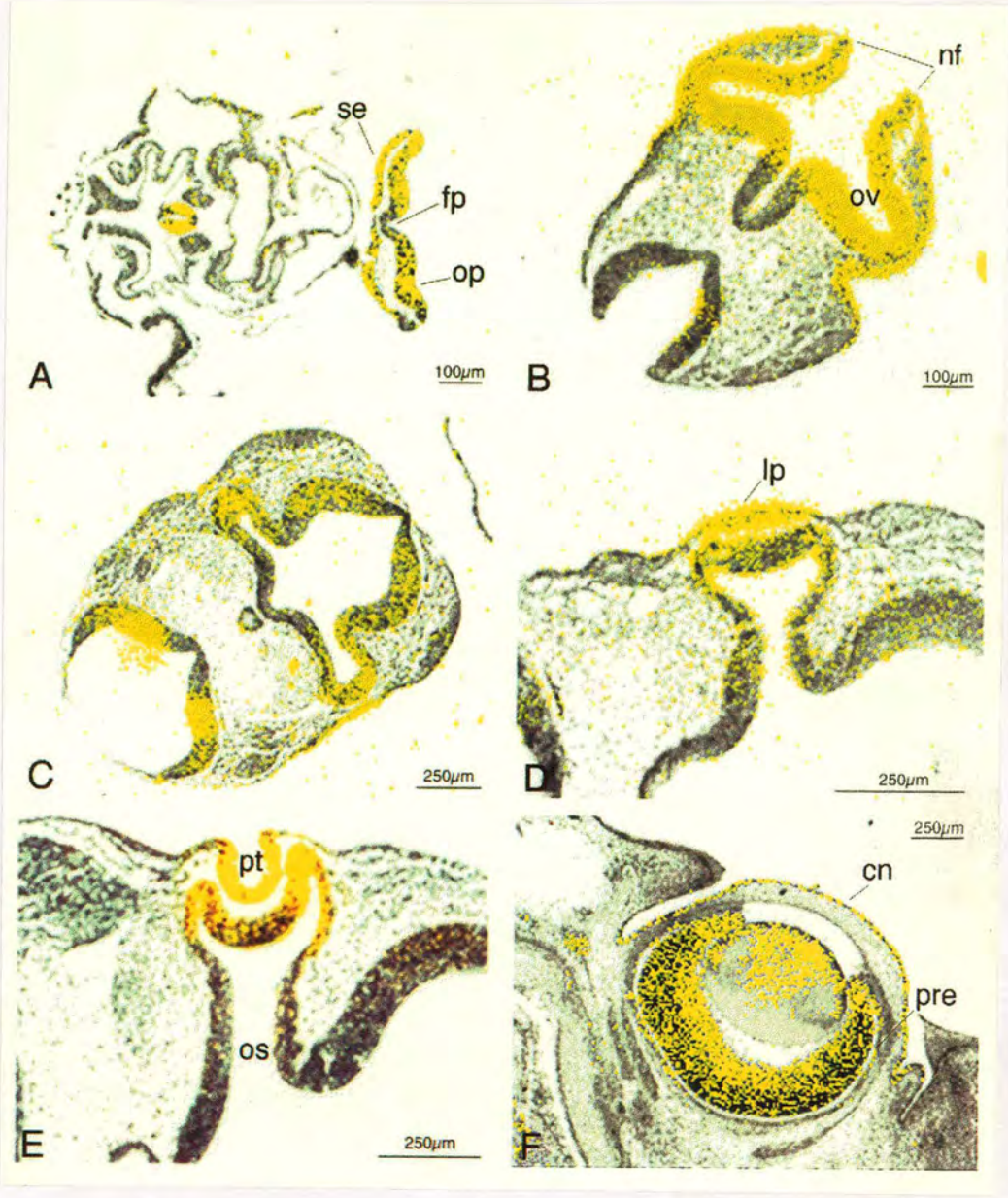

Figure 3.11

Expression of *Pax-6* mRNA, yellow, during early eye development in wild-type mice. Radioactive *in-situ* hybridisation on transverse sections at embryonic ages E8.0, (A); E8.5,(B); E9.25, (C); E9.5, (D); E10.5, (E) and E15.5, (F). Surface ectoderm (se), optic pit (op), floorplate (fp), neural folds (nf),optic vesicle (ov), lens placode (lp), lens pit (pt), optic stalk (os), retinal pigment epithelium (pre), cornea (cn).

3.3 Gene expression in the eyes of *Sey/Sey* embryos.

3.3.1 *Pax6* and *Tyrp2* expression domains in optic vesicles of *Sey/Sey* embryos.

To characterise the structures formed by the optic vesicle in the absence both of functional PAX6 protein and of lens, histological analysis was combined with *in situ* hybridisation using probes for gene transcripts that normally display region-specific expression patterns within the optic vesicle. The single base change in the *Sey* allele, whilst predicted to result in a truncated PAX6 protein (Hill *et al.,* 1991), will not affect the *in situ* hybridisation assay for *Pax6* mRNA expression, thus *Pax6* can also be used as a marker in this way. Expression of the tyrosinase-related protein gene *Tyrp2,* was also used as an early optic vesicle marker that becomes restricted to the RPE, the pigmented retinal epithelium layer of the optic cup, by about E10.5, (Steel *et al.,* 1992). *Sey/Sey* and control embryos were hybridised with antisense probes for *Pax6* (stages E8.0 to E15.5) or *Tyrp2* (stages E11.5-E12.5)

Pax6 expression in *Sey/Sey* optic vesicles is initially indistinguishable from that in +/+ and *Sey*/+ littermates (Fig. 3.15 B). As optic vesicle abnormalities become apparent in *Sey/Sey* mutants, *Pax6* expression retains features seen in the expression patterns of littermates. Thus at E9.5 to E10.5, *Pax6* expression is strongest in most distal structures (Fig 3.15 C). Later still, *Sey/Sey* optic vesicles form distinct optic stalk-like and bi-layered optic cup-like structures, shown at E15.5 in Figures 3.12 A,B. As for normal eyes, it is the two neural ectoderm layers of the optic-cup like structure that express *Pax6* mRNA (Fig. 3.12 C) and there is little expression in the optic stalk. Using the *Tyrp2* probe there is typically a small domain of intense signal in the most distal regions of the *Sey/Sey* optic vesicle (Fig. 3.12 G). Where the optic

vesicle forms a cup, *Tyrp2* transcripts are most abundant at the rim of this cup , but are present in both layers. Unlike the clearly differentiated pigmented retinal epithelium and neural retina in the optic cups of normal embryos, the two neuroepithelial layers, in those structures in *Sey/Sey* embryos that resemble cups, are of similar thickness and appearance (Fig. 3.12 E,G). In these structures, *Tyrp2* is almost exclusively expressed within the larger, but still distally restricted, *Pax6* expression domains (Fig. 3.12 E). This is in contrast to normal embryos, where there is a considerable region of pigmented retinal epithelium that expresses *Tyrp2,* (Fig. 3.12 F) but not *Pax6,* (Fig. 3.12 D).

Figure 3.12 (see following page)

The optic vesicle phenotype of *Sey/Sey* animals analysed by gene expression patterns. Optic vesicle phenotype of *Sey/Sey* embryos at E15.5, (A-C).(A) Transverse sections through optic vesicle (ov), showing separation from skin by intervening tissue. (B) Detail of bi-layered optic cup structure (oc), and optic stalk (os). (C) *Pax-6* mRNA expression, predominantly in cup-like structure. (D-G) Expression domains of *Pax-6* mRNA (D,E), and *Trp-2* mRNA (F,G) in transverse sections of control (D,F) and *Sey/Sey* (E,G) eyes at E12.5. Developing pigmented retinal epithelium (pre), developing neural retina (nr). Anterior is to the left.

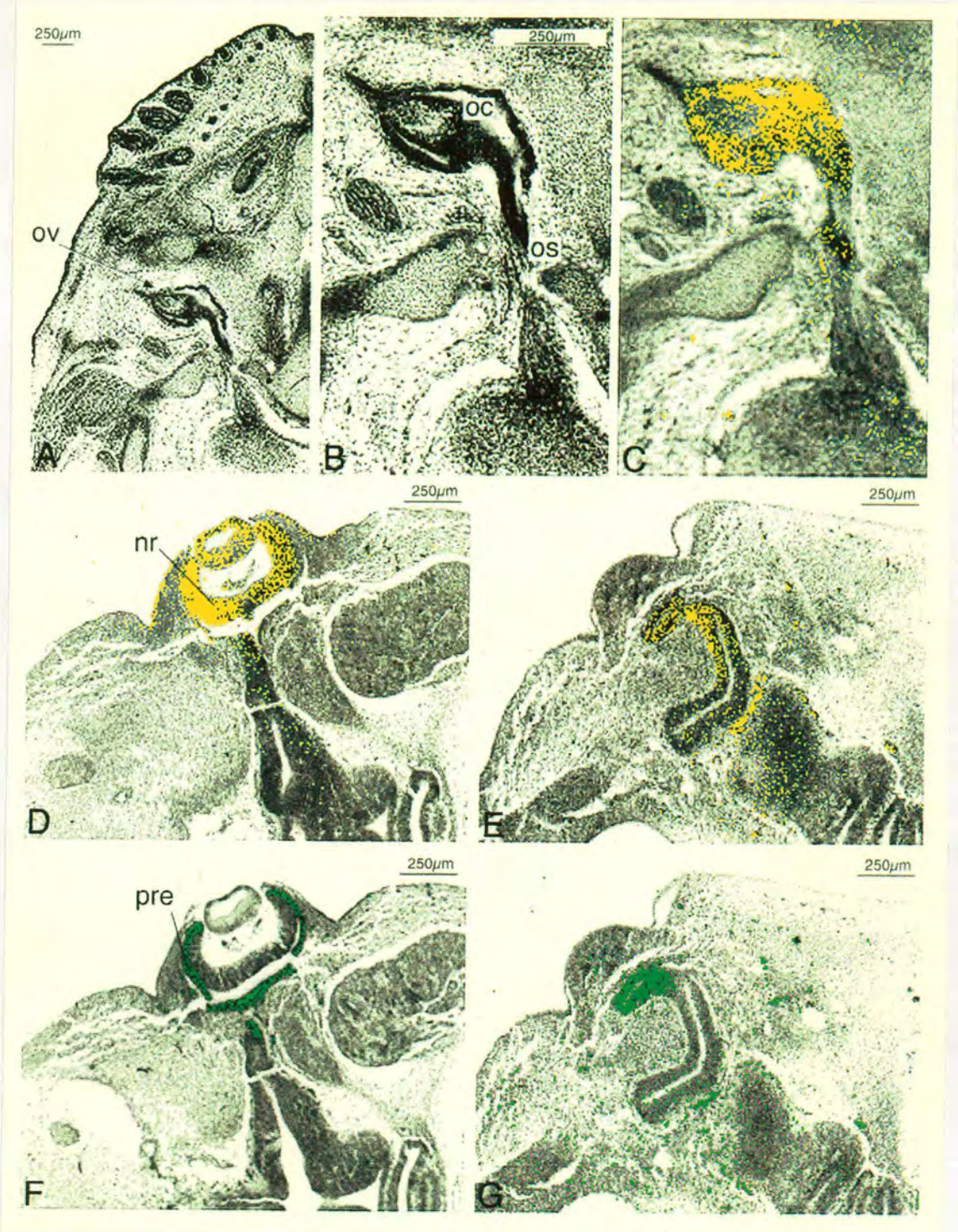

Figure 3.12 (see preceding page for legend)

3.3.2 Distinct layers in *Sey/Sey* distal optic vesicles.

Expression studies and morphological analysis above suggest that at E11.5 there is little or no distinction between the layers of the optic cup-like structures of *Sey/Sey* embryos. Later however differences are apparent, and are shown at E15.5 in Figure 3.13. The outer layer of this cup like structure has well defined boundaries and a closely packed structure. The inner layer is thinner, and has less well defined boundaries, being made up of more loosely packed cells. The transition zone between the two layers is also more loosely packed than the outer layer and contains cells with a rounded rather than bipolar morphology. The loose packing and absence of well defined boundary on the inner layer may reflect a loss of tissue integrity reminiscent of degeneration.

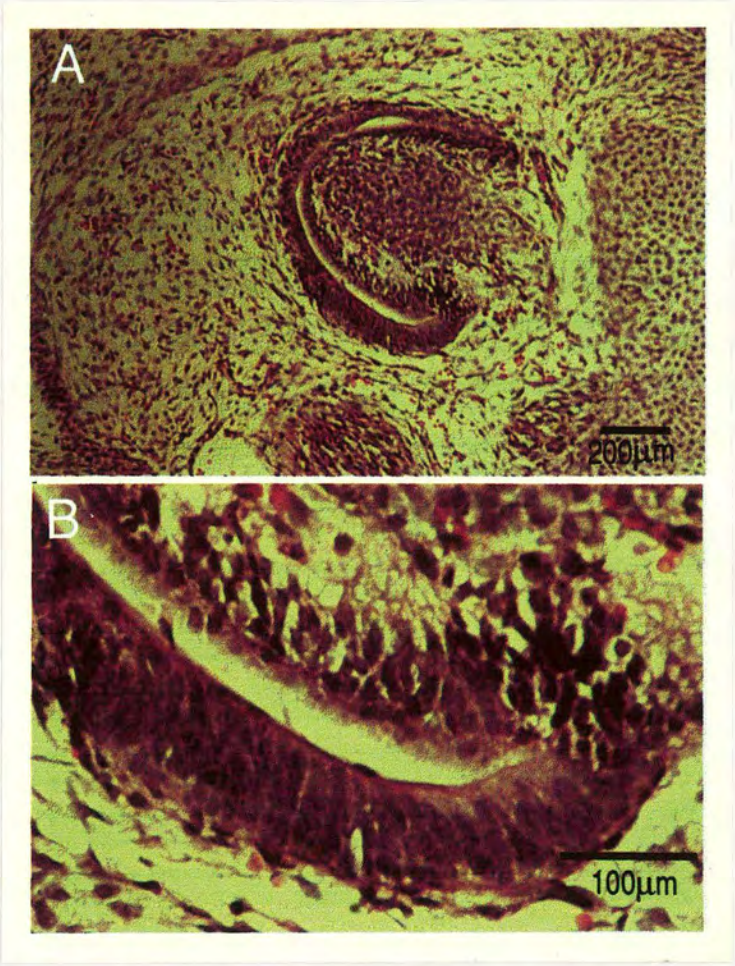

Figure 3.13

Distinct layers in the *Sey/Sey* optic cup at E15.5 . Overview (A) and detail (B) of the cup-like structure formed by the distal optic vesicle in *Sey/Sey* embryos. The two layers have distinct morphologies at this stage. The outer layer contains tightly packed neuroepithelial cells and has well-defined boundaries. The inner layer consists of more loosely-packed cells. The boundaries of the inner layer, both with the lumen of the optic vesicle, and with underlying mesenchyme, are poorly-defined. The junction between the layers contains many small, intensely-staining, cells with a rounded morphology.

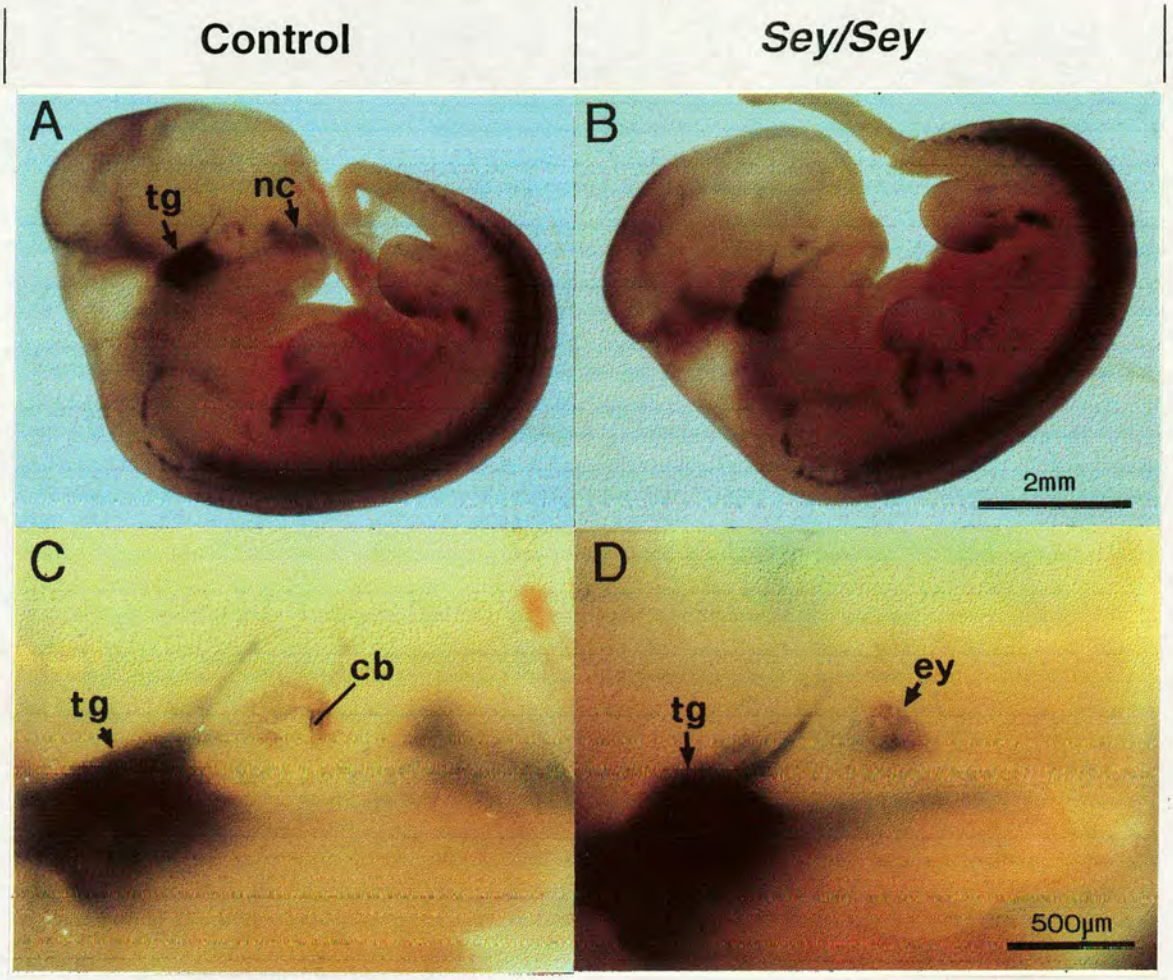

Figure 3.14

Msx1 transgene expression in the eye. Staining with X-gal of an *Sey/Sey* embryo (B, D) and a littermate (A, C) at E11.5, carrying an *Msx1 lacZ* reporter transgene. (A, B) *Sey/Sey* embryo (B) retains most of the normal pattern of *Msx1* transgene expression seen in (A), but lacks the expression associated with the nasal cavities (nc). (C, D) In the eye region, transgene expression (blue) is present in the control (*Sey*/+) embryo (C), in the anterior margin of the dorso-nasal retina, in line with the timing and location of expression in the presumptive ciliary body of +/+ animals. The lighter, brown staining in the eye is retinal pigment. In the *Sey/Sey* embryo (D) there as a cluster of blue staining cells below the surface in the eye region (ey), in the position of the distal optic vesicle. Brown pigment is also visible in the eye region. (tg) trigeminal nucleus; (nc) nasal cavities; (cb) ciliary body *Msx1* transgene expression; (ey) eye region of *Sey/Sey* embryo.

3.3.3 Expression of an *Msx1* transgene in *Sey/Sey* eyes.

The *Msx1* gene is expressed in the presumptive ciliary body prior to its morphological appearance (Monaghan *et al*, 1991). Ciliary body expression is also a feature of the *Msx1* transgene used in the analysis of nasal development, but in this case the lacZ expression is present well before expression of the endogenous gene can be detected by *in situ* hybridisation (Davidson, D. R., personal communication). In normal animals this ciliary body lacZ expression first appears around E11.5 on the dorso-nasal region of anterior optic cup (Fig 3.14 A, B) This subsequently spreads to the remaining dorsal, and then ventral, presumptive ciliary body. In *Sey/Sey* animals most aspects of the *Msx1* expression pattern are identical to littermate controls, with the exception of the expression in nasal cavities that will be described later. In the eye region, small patches of blue staining cells are present close to but below the surface, in the position occupied by the distal optic vesicle at this age.

Pigmented animals were used as parents for these crosses and retinal pigment, which appears as brown in these preparations, is apparent in the control embryos. *Sey/Sey* embryos also have some brown pigment, suggesting that they produce some retinal pigmented epithelium.

3.3.4 Surface ectoderm *Pax6* mRNA expression in homozygous mutant mice

PCR genotyping (see materials and methods) allows identification of *Sey/Sey* embryos prior to the appearance of morphologically recognisable defects. As mentioned above, the single base change in the *Sey* allele does not affect the *in situ* hybridisation assay for *Pax6* mRNA expression, thus it has been possible to analyse *Pax6* expression in early *Sey/Sey* embryos.

Genotyped embryos were sectioned and hybridised with antisense *Pax6* probe. *Sey/Sey* embryos showed no differences from the normal pattern of expression at E8.0 or E8.5 (Fig. 3.15 A,B). By E9.5- E9.75 however, when in normal embryos expression was confined to around the lens and nasal placodes, no expression was detected in the surface ectoderm anywhere in the heads of homozygous mutants, including the eye region (Fig. 3.15 C).

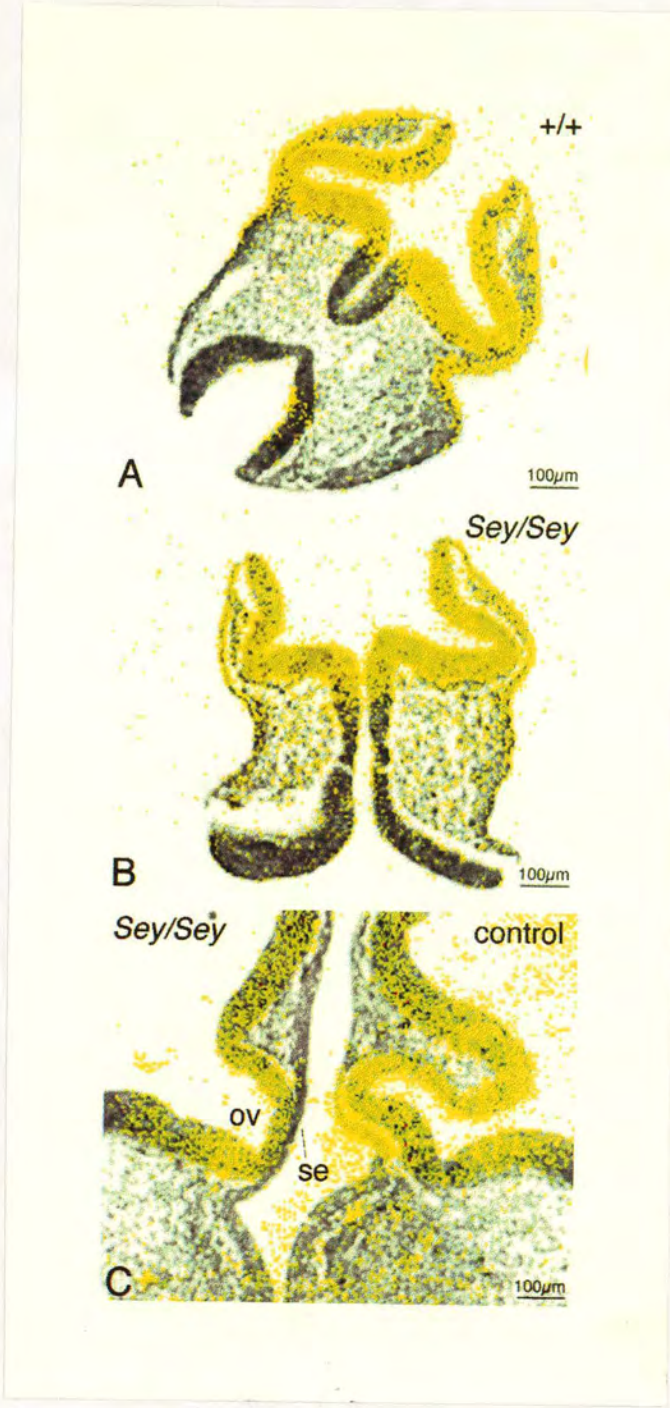

Figure 3.15

Pax6 expression in the eyes of *Sey/Sey* embryos. *Pax-6* mRNA expression, yellow, in wild-type embryo (A) and *Sey/Sey* embryo (B) at E8.5. *Sey/Sey* embryos at this stage have the same *Pax-6* mRNA expression pattern as their littermates. By E9.75 (C) surface ectoderm (se) expression adjacent to optic vesicle (ov) in littermate (right) is absent from *Sey/Sey* embryo (left).

3.4 Studies on nasal development

3.4.1 *Pax6* expression and nasal placode development

Pax6 mRNA (Fig. 3.16 A) is present in the nasal placodes; though at a lower level than in lens placodes. This expression continues in placodal epithelium during the formation of nasal pits (Fig. 3.16 B) and subsequently expression is detectable in the developing olfactory epithelium, as has been previously reported by Walther and Gruss (1991). No nasal placodes could be detected on serial sections through the nasal region of *Sey/Sey* embryos at E9.75, whereas littermates at this stage have well formed nasal placodes undergoing the first stages of invagination to form a nasal pit. The nasal placode *Pax6* mRNA detectable in littermates (Fig. 3.16 C) is absent from *Sey/Sey* embryos at this stage (Fig. 3.16 D) although *Sey/Sey* embryos retain expression in the neuroepithelium of the developing forebrain.

3.4.2 *Msx1* transgene expression in the nasal placode region

In +/+ and +/*Sey* mice carrying the *Msx1* transgene, lateral placodal epithelium cells express the lacZ marker at a high level, whereas non-placodal ectoderm cells, underlying mesenchyme and medial nasal placode cells do not. This specific expression is detectable at E9.5 and develops into an arc of strong expression in the placodal ectoderm of the prospective lateral nasal process by E10.5 (Fig 3.17 A,B). X-gal staining of *Sey/Sey* embryos (Fig 3.17 C) and littermates (Fig 3.17 B) from E9.0- E11.5 showed that this early marker for nasal placode differentiation is never expressed in the absence of functional PAX6 protein.

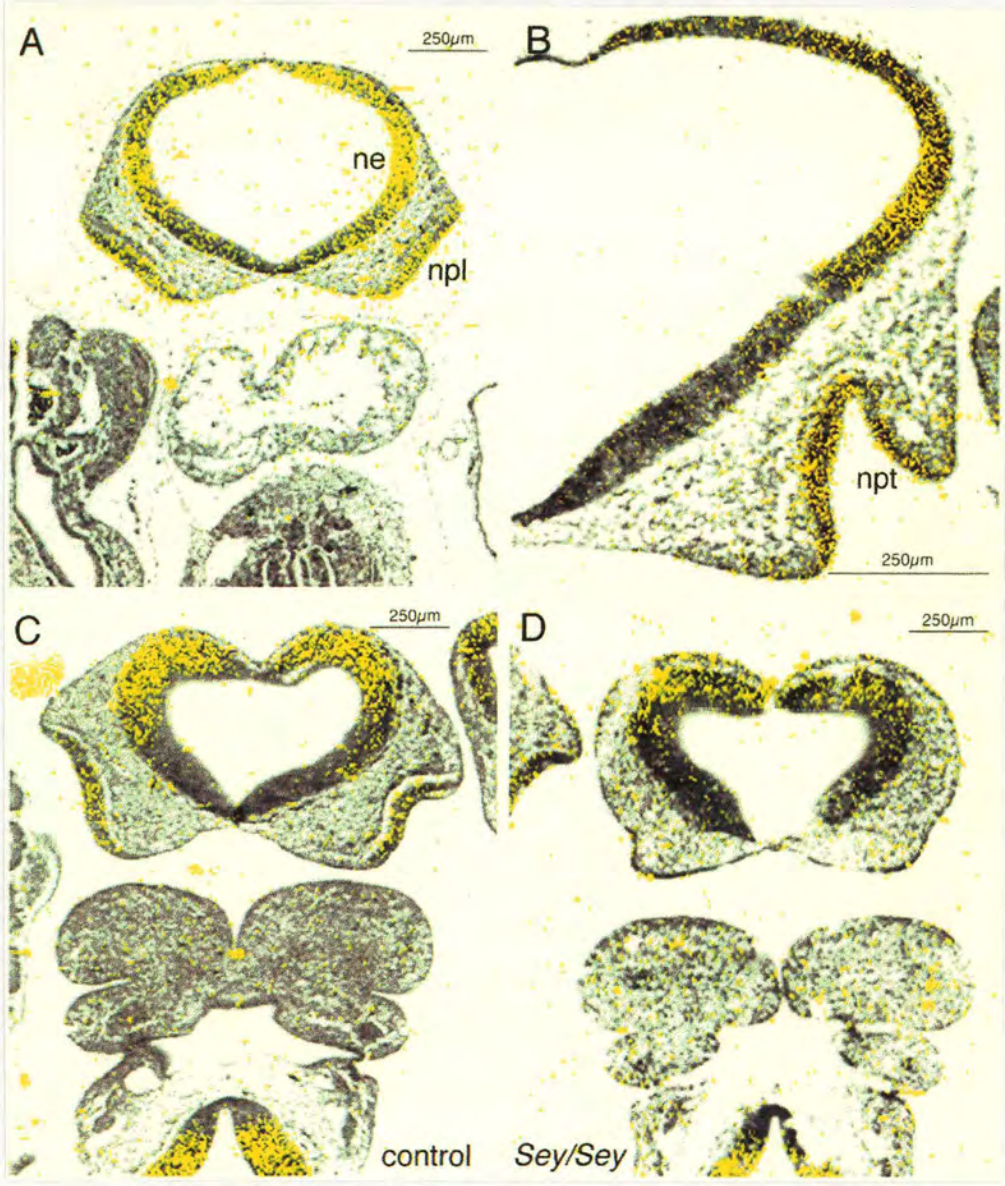

Figure 3.16

Pax6 expression and the nasal placodes. Frontal sections through the nasal region showing *Pax-6* mRNA expression, yellow, in normal embryos at E9.5 (A) and E10.5 (B). Absence of nasal placode and nasal region expression at E9.75 from *Sey/Sey* embryo (D), compared with control embryo (C). Nasal placode (npl), nasal pit (npt), neuroepithelium of prosencephalon (ne).

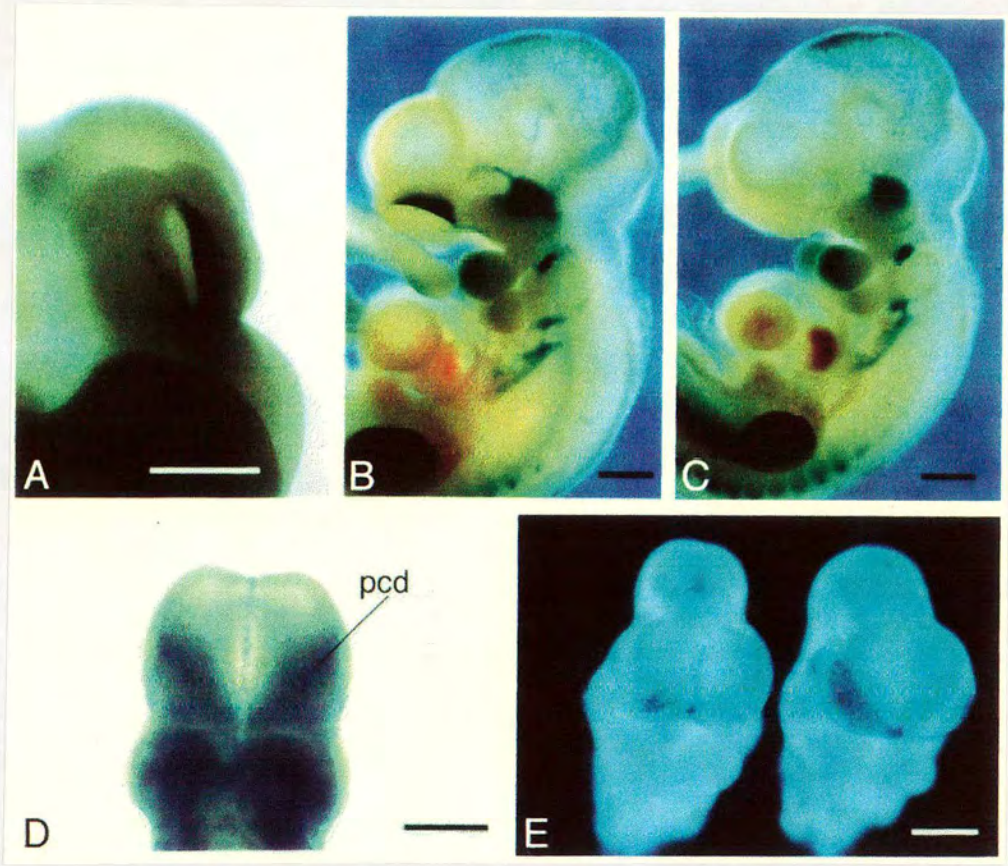

Figure 3.17.

Msx1 transgene expression and Nile Blue Sulphate staining in the nasal region of *Sey/Sey* mice and their littermates. Expression of the *Msx-1* transgene ΔH6 at E10.5 (A-C). Normal lateral placodal epithelium expression (A). Control embryo, (B). *Sey/Sey* embryo, (C). Nile Blue Sulphate (NBS) staining for programmed cell death (D-E). Arc of NBS-staining, programmed cell death (pcd), around rim of normal placode in (D). (E): NBS staining in *Sey/Sey* embryo (left) and control embryo (right). All scale bars represent 1 mm.

3.4.3 Cell death in nasal placode region.

In normal embryos, a narrow arc of cell death appears around the edge of the developing nasal placode prior to invagination (Fig. 3.17 D). Patterns of cell death, detected by Nile Blue Sulphate (NBS) staining, can be used as markers to study the fate of cells in the nasal region in *Sey/Sey* embryos. In contrast to the restricted arcs of NBS staining cells in normal embryos, diffuse patches of NBS stained cells were found in the nasal region of E9.25-E9.75 *Sey/Sey* embryos (Fig. 3.17 E). The number of stained cells varied considerably from embryo to embryo, and between left and right sides of the same embryo, but consistently these patches were diffuse. Analysing the cell death patterns at E10.5, the majority of NBS staining regions are found to be identical in *Sey/Sey* embryos and their littermates, but there are three differences. 1. Cell death normally occurs at the point of fusion of the lateral and medial nasal processes. The position of the processes formed in *Sey/Sey* embryos and the cell death pattern on them identifies these as a medial nasal processes, the cell death domains of the lateral nasal processes being absent. This supports the conclusions of previous scanning electron microscopy studies by Heinzmann *et al.,* (1991), that the lateral nasal processes are absent in *Sey/Sey* embryos at this stage. 2. In 10.5 day *Sey/Sey* embryos, bilateral ectopic bands of subectodermal NBS staining are observed over the anterior forebrain. This may identify a population of midbrain neural crest cells that, in homozygous rat *Smalleye* (*rSey*) embryos, fail to reach the nasal region (Matsuo *et al.,* 1993), and may die. 3. The normally narrow band of NBS staining along the midline of the brain is broadened in *Sey/Sey* embryos into a diamond shape patch over the telencephalon-diencephalon boundary.

CHAPTER 4

Discussion of the involvement of the *Pax6* gene in eye and nasal development

4.1 Early optic vesicle abnormalities in *Sey/Sey* embryos.

The optic vesicle and surface ectoderm both express *Pax6* during normal development, and both show abnormalities in *Sey/Sey* mice. In normal eye development, there are multiple interactions between these two tissues. The optic vesicle is thought to be important for correct positioning and growth of the lens and its orientation (Yamamoto, 1976; reviewed by Grainger, 1992). Equally the developing lens appears to be important for growth of the retina. (Coulombre and Coulombre, 1964).

The first developmental defects of *Sey/Sey* embryos detected in this study are the abnormal shape of the brain and optic vesicle. The failure of the optic vesicles to constrict proximally occurs before the time of normal lens development and may reflect a requirement for PAX6 in the neural ectoderm. The subsequent absence of lens may contribute to the later distal distortion of *Sey/Sey* optic vesicles, but this phenotype might also result from a lack of functional PAX6 in the distal optic vesicle, where *Pax6* mRNA is most strongly expressed.

Compared with littermates, optic vesicles of *Sey/Sey* from E9.5-E11.5 have a more uniform lumen width along their proximal-distal axis. *Sey/Sey* optic vesicles (E11.5 to E15.5) do eventually form structures reminiscent of both optic cup and optic stalk. These *Sey/Sey* structures vesicles retain some features of normal patterning, despite morphological abnormalities. *Tyrp2* which in normal eyes is expressed only in the most distal optic stalk and the RPE layer of the optic cup, retains this distally-restricted expression in *Sey/Sey* optic vesicles. As found in normal eyes, *Pax6* transcripts in *Sey/Sey* optic vesicles are also most abundant in

distal structures and are present in more extensive domains than those of *Tyrp2*. The *Msx1* transgene provided a third marker normally expressed in the distal optic vesicle. Expression of this transgene in the eye region of *Sey/Sey* embryos is first detected at the same time as it is in control embryos, and is present in the position of the distal optic vesicle, suggesting that some aspects of the normal spatio-temporal pattern of expression of this marker are also retained.

Tyrp2 and *Pax6* expression also highlight some abnormal features of *Sey/Sey* optic vesicles. Schmahl *et al.*,(1993) suggested that the optic vesicle abnormalities could result from a disruption of proliferation or differentiation choices in the distal optic vesicle. My findings support this suggestion. The small size of the expression domains of *Tyrp2* and *Pax6* in *Sey/Sey* embryos may indicate that growth of the distal optic vesicle fails to keep pace with the development of other structures. This may explain the increasing separation of optic vesicle from the surface ectoderm. The two layers of the cup-like structure in *Sey/Sey* optic vesicles are morphologically similar and have similar expression characteristics, suggesting that differentiation of the developing cup into distinct layers is disrupted. Since there is no clear equivalent of the RPE, differentiation towards this cell type might be particularly affected in *Sey/Sey* mice. At later stages (by E15.5) a distinction between the two layers is apparent, although one layer may be degenerating by this time. A complete blockage of RPE production appears unlikely, however, since pigmented cells are present in the eye region of *Sey/Sey* embryos carrying the *Msx1* transgene. This contrasts with a complete absence of a contribution to the RPE by *Sey/Sey* cells in chimeras (Jane Thornbury, personal communication).

Whilst the absence of lens in *Sey/Sey* mice complicates the identification of roles for *Pax6* within the optic vesicle, it also allows *Sey/Sey* mice to be used to study the influence of the lens on optic vesicle development. Thus, those aspects of proximo-distal patterning of the optic vesicle that are retained by *Sey/Sey* embryos do not depend upon the presence of a lens adjacent to the distal end of the optic vesicle.

4.2 PAX6 in lens development

4.2.1 Timing of PAX6 action in lens formation.

Lens 'induction' is a multi-step process (Jacobson, 1966; Grainger *et al.*,1988). The initiation of the lens formation pathway involves a series of inductive interactions prior to the contact between the surface ectoderm and the optic vesicle. Surface ectoderm passes through a progression of states of competence to respond to the different lens-inducing signals (Karlinen-Jääskeläinen, 1978; Henry and Grainger, 1987; Henry and Grainger, 1990; Serventnick and Grainger, 1991; reviewed by Jacobson and Sater, 1988; Grainger, 1992). Although contact with the optic vesicle was long thought to be both necessary and sufficient to induce surface ectoderm to form lens (reviewed by Saha *et al.,* 1989), it has been shown that the optic vesicle is not essential for initial formation of lens but can nevertheless induce lenses from ectoderm with lens-forming potential (Henry and Grainger, 1990) and is important for maintenance and growth of the lens (Saha *et al.,* 1989).

Within the process of lens development, PAX6 must act prior to, or at, the time of placode formation, i.e. before E9.5 in the mouse. Studies in amphibians suggest that the very earliest stages of lens induction occur around the time of gastrulation (Jacobson, 1966; Henry and Grainger, 1987). If comparable processes

occur in mammalian eye development they are unlikely to involve PAX6, since *Pax6* is only expressed from the time, (E8.0 in mouse), when the optic pits first appear. (Walther and Gruss, 1991; Krauss *et al.,* 1991a; Krauss *et al.,* 1991b; Puschel *et al.,* 1992). Since surface ectoderm in *Sey/Sey* embryos fails to attain the earliest recognisable stage of lens formation, use of these animals does not address possible roles for *Pax6* later in lens development. Nevertheless, PAX6 is necessary for normal lens formation during the time, E8.0 - E9.5, between the first appearance of *Pax6* expression and lens placode formation.

4.2.2 PAX6 and lens determination.

In normal development of the mouse eye, broad domains of *Pax6* mRNA expression in head surface ectoderm are downregulated, with expression being maintained in the developing lens placode. In the chick, the ability of isolated ectoderm to differentiate into lens in culture is initially a property of head ectoderm over a broad domain which, with time, also becomes restricted to the lateral regions adjacent to the optic vesicles (Barabanov and Fedtsova 1982). Therefore, the surface ectoderm expression of the *Pax6* gene and the *Sey/Sey* mutant phenotype are consistent with a role for PAX6 in lens determination.

Tissue recombination experiments using the rat *Small eye* (Fujiwara *et al.,*1994) found that lens formation depended upon the genotype of the surface ectoderm used and was independent of the genotype of the optic vesicle. Thus *rSey/rSey* surface ectoderm never formed lens, whereas +/+ and *rSey*/+ surface ectoderm could form lens, even when cultured with *rSey/rSey* optic vesicle. It is possible that these results reflect a failure of signalling in-vivo prior to the 20 somite

stage when the recombinations were performed, but they are equally consistent with a requirement for functional PAX6 within the surface ectoderm in order to form a lens placode and lens. Moreover they further limit the role of the optic vesicle in the development of the homozygous mutant phenotype, as *rSey/rSey* optic vesicles can support the later development of the lens.

4.2.3 *Pax6* expression and ability to transdifferentiate to lens

Some non-lens tissues that express *Pax6* are able to transdifferentiate into lens. The prospective pineal gland expresses *Pax6* (Walther and Gruss, 1991), is multipotent, and has the capacity to form lens (Watanabe *et al.,* 1992). Similarly embryonic retina, iris and pigmented retinal epithelium at the anterior of the eye all express *Pax6* mRNA and all have the ability to transdifferentiate into lens, (reviewed by Okada, 1991). *Pax6* expression may be a prerequisite for lens formation, and in non-lens tissue, may reflect a lens-forming ability that is normally overridden by diversion to other pathways.

The possibility that the failure of lens formation in *Sey/Sey* embryos results entirely from a disruption of an interaction with the optic vesicle cannot be ruled out. An alternative explanation, PAX6 involvement in lens determination, is supported by the association of *Pax6* expression with ability of surface ectoderm to differentiate into lens, by the rat *Small eye* tissue recombination results and by the relationship between *Pax6* expression and ability to transdifferentiate into lens. In addition, an activity of PAX6 independent of the influence of the optic vesicle is revealed by down-regulation of *Pax6* mRNA in *Sey/Sey* surface ectoderm.

4.3 Down-regulation of *Pax6* mRNA in *Sey/Sey* surface ectoderm.

Activation of *Pax6* mRNA in *Sey/Sey* embryos is normal, but at the time when most surface ectoderm normally ceases to express *Pax6,* the turn-off mechanism appears to extend to the whole surface ectoderm.

This extended down-regulation could be explained in a number of ways but the observation that *Pax6* mRNA continues to be expressed in normal chick surface ectoderm in the absence of an optic vesicle (Li *et al.,* 1994), argues against failed signalling from optic vesicle and against a negative influence on *Pax6* expression from mesenchymal-like cells gaining access to placode region surface ectoderm. I suggest a role for PAX6 as an effector of its own expression in placode-forming ectoderm. Plaza *et al.,*(1993) found a PAX6 binding site in the quail *Pax6* promoter through which PAX6 up-regulated its own transcription in transfection assays, so the PAX6-dependent regulation observed in this study may reflect direct auto-regulation.

4.4 Nasal placode formation and nasal processes.

Both nasal cavities and lens develop from ectodermal placodes. There are extensive similarities between the nasal lens placodes in their *Pax6* expression and *Sey/Sey* mutant phenotype. Like the lens placode, the nasal placode normally expresses *Pax6* mRNA and fails to form in *Sey/Sey* mutants. Similarly *Pax6* mRNA expression turns off throughout the nasal region in *Sey/Sey* embryos. These results are consistent with the suggestion of Hogan *et al.,* (1986), that the eye and nasal phenotype of *Sey/Sey* mice might stem from a common defect in the formation or early differentiation of these placodes.

The absence of nasal region *Msx1* transgene expression in *Sey/Sey* embryos allows us to speculate that *Msx1*, or genes regulating it, could be targets for PAX6 in the nasal region. More concretely, absence of this expression shows that ectoderm in the nasal region of *Sey/Sey* embryos not only fails to attain the morphology characteristic of a placode, but also lacks gene expression normally associated with the nasal placode. Characteristic domains of morphological cell death, detected by Nile Blue Sulphate staining, are present in the nasal region of *Sey/Sey* embryos and control animals but in *Sey/Sey* embryos the domains are disorganised. Presence of these domains indicates that nasal region ectoderm is distinct from other head ectoderm, and so suggests that localisation of the nasal territory need not involve PAX6 expression. Instead, PAX6 may play a role in the transition from ectoderm to placode. From the similarity between lens and nasal placodes, in their normal development, *Sey/Sey* phenotype and *Pax6* expression, I expect the requirement for PAX6 in lens placode development to also be in the transition from ectoderm to placode.

4.5 Information on complex developmental systems from the analysis of *Small eye*.

The defects of *Small eye* mice provide a genetic model system for the analysis of craniofacial development (Hogan *et al.,* 1988). They are of potential use in the analysis of a wide range of problems, since many structures and cell types are affected, including the brain, nasal cavities, lens, iris, cornea and, migrating neural crest (Hogan *et al.,* 1986; 1988; Jordan *et al.,* 1992; Schmahl *et al.,* 1993; Matsuo *et*

al., 1993; Hanson *et al.,* 1994; Anthony LaMantia, personal communication). This very range of defects, however, makes it difficult to draw unequivocal conclusions about the processes of normal development from *Small eye,* since so many processes may be being disrupted simultaneously. Nevertheless, the analysis in the present current study provides information on the location and timing of defects in *Sey/Sey* embryos, suggests processes that might be affected, indicates cases where abnormalities may be independent of known interacting tissues and, importantly, describes features of normal development that are retained, despite the severe perturbation of cranio-facial development.

The analysis of *Small eye* mice demonstrates that the formation of the conjunctival sac and eyelids proceeds on schedule in the absence of an eyeball. Moreover, the initial development and orientation of the ocular muscles also appears normal in *Sey/Sey* embryos although it is unclear what tissue they ultimately terminate within.

By PCR genotyping of embryos it has been possible to trace the abnormalities of *Small eye* further back in development than previously possible in the mouse. This revealed a defect of the proximal optic vesicle that is probably independent of the presence of a lens. Conversely, the down-regulation of *Pax6* expression in surface ectoderm suggests a function for PAX6 within the placode-forming ectoderm that is independent of the optic vesicle. Another significant finding for the understanding of normal eye development, is that aspects of normal patterning within the optic vesicle occur in *Sey/Sey* embryos, and so must be independent of the presence of a lens.

Analysis of a complex organ, such as the eye, using the *Small eye* mutant has

thus provided information, not only about the tasks that a single gene may perform in different components of this structure, but also about the ways in which these components may interact or act independently.

CHAPTER 5

Results of studies aimed at identifying roles for *Pax6* in the developing brain.

5.1 Experiments examining the roles of *Pax6* in brain development.

The pattern of *Pax6* mRNA expression in the developing CNS was examined in wild-type mouse embryos from E8.5 to E17.5 by *in situ* hybridisation. Particular attention was paid to the developing forebrain, since developmental abnormalities of the forebrain in *Small eye* animals had been reported, including absence of olfactory bulbs (Hogan et al, 1986) and abnormalities of neuronal migration in the developing cerebral cortex (Schmahl et al, 1993). *Pax6* expression was related to the expression of the *steel* and *LH-2* genes and morphological differences between *Pax6* expressing and non-expressing cells were identified.

A defect of early forebrain development was identified in *Sey/Sey* animals. In addition, to assist with a comparison of *Small eye* defects and *Pax6* expression, the work of Schmahl et al, (1993) using Sey^{Neu} animals was extended to animals carrying the *Sey* allele.

The early forebrain and optic vesicle morphology abnormalities that had been identified were characterised by light microscopy of dissected embryos, histological analysis and *in situ* hybridisation, in which *Pax6* expression was examined in *Sey/Sey* mutants. This suggested there might be a defect in antero-posterior patterning of the diencephalon in *Sey/Sey* animals, which was tested by the use of scanning electron microscopy to examine the ventricular surfaces the brain.

5.2 *Pax6* mRNA expression in the developing nervous system.

5.2.1 Variation in the pattern of *Pax6* mRNA expression along the antero-posterior axis of the CNS in wild-type mice.

The pattern of *Pax6* mRNA expression at all stages examined was complex. There is expression of *Pax6* throughout the length of the CNS but the abundance of transcripts, the dorsal ventral boundaries of expression and the time at which expression is first detected all vary along the antero-posterior axis. Figures 5.1 A and 5.1 B show wholemount *in situ* hybridisations using a *Pax6* probe from E8.75 to E9.25 and at E10.5 respectively. At these stages there is *Pax6* expression in the developing spinal chord, hindbrain and forebrain, but no extensive expression in the developing midbrain.

The variation in abundancy of *Pax6* transcripts along the antero-posterior axis is readily apparent by E8.75 (Fig. 5.1). At this stage there is already a clear boundary between presumptive forebrain which expresses *Pax6* mRNA and presumptive midbrain, which does not. In addition a region of weaker *Pax6* mRNA expression separates this strong expression near the boundary with the midbrain from the strong expression adjacent to, and in, the optic vesicles. As the diencephalon becomes morphologically divided into neuromeric units (Figdor and Stern, 1993), changes in the levels of *Pax6* expression are observed to coincide with the boundaries between these neuromeres, such as that between posterior and anterior parencephalon (between dorsal and ventral thalamus). This relationship is shown at E10.5 in Fig. 5.1C. At this stage there is strong *Pax6* expression both in the developing ventral

thalamus and in the synencephalon. In intervening segments, *Pax6* expression levels are lower, but there is some strong expression closer to the midline than shown in this parasagital section. In particular there is strong expression in the prospective epiphysis.

The dorso-ventral domains of expression in the forebrain are different from those elsewhere. Elsewhere in the nervous system a useful morphological landmark for descriptions of dorsal and ventral is the sulcus limitans, which divides the walls of the neural tube into alar and basal plates. The rostral limit of the sulcus limitans is in the posterior diencephalon, but an imaginary rostral continuation of the sulcus limitans may be constructed by taking the level of maximum width of the third ventricle. If this is done, then *Pax-6* expression within the diencephalon is found to be almost exclusively dorsal (or anterior, due to the flexure of the brain) of this level (Fig. 5.2 C, Fig. 5.7 D). This is in contrast to the hindbrain and developing spinal chord in which, consistent with a previous report (Walther and Gruss, 1991) *Pax6* expression was found to be progressively restricted to the cells of the ventral ventricular zone. One common feature along the antero-posterior axis, however, is that at no stage examined were *Pax6* transcripts detected in the floor plate (or ventral midline) in any region of the CNS.

There were differences in the time at which *Pax6* expression could first be detected. *Pax6* is extensively expressed in the CNS at E8.0 and then becomes more spatially restricted in expression with time. In the midbrain, however, there are narrow ventro-lateral domains of expression that are first detected at E10.5 (long arrow in figure 5.1 C), and later, at E11.5, they are apparent as blocks of strongly

expressing cells which extend neither to the ventricular nor to the pial surfaces (Fig. 5.1 D).

5.2.2 Domains of *Pax6* expression in the telencephalon of wild-type mice.

The topological notation of Kuhlenbeck (reviewed by Kuhlenbeck, 1973), divides the vertebrate forebrain on morphological grounds into numbered pallial (or dorsal) domains (D) and basal domains (B) some of which are shown in Figure 5.2 A. These domains have distinct *Pax6* expression characteristics (shown in caudal section at E10.5 in Fig. 5.2 B and in a transverse section at E11.5 in Fig. 5.2 C). Using this notation for both the stages illustrated, there is *Pax6* expression in D_3, stronger expression in D_2 and generally lower levels of expression in the lateral ganglionic eminence, D_1. The basal regions B_1 and B_2, collectively forming the medial ganglionic eminence, do not express *Pax6*, and neither does the B_3 region, near the midline (not shown). Stronger expression than any of these domains was found in the telo-diencephalic sulcus, the fold of tissue at the boundary betweeen diencephalon and telencephalon (Fig 5.2C).

As the neural ectoderm of the telencephalon forms distinct ventricular, intermediate and marginal zones, *Pax6* expressing cells are generally absent from the marginal zone (Fig 5.2 C). One exception to the absence of expressing cells from the marginal zone is the narrow band of signal seen in the ventro-lateral telencephalon (shown at E10.5 in Fig 5.2 D and at E11.5 in Fig. 5.2 B) which may correspond to the presumptive piriform cortex. The posterior poles of the telencephalon express *Pax6* particularly strongly (Figures 5.4 A-B, 5.7 G, 5.3 A), and here, too the expressing cells can be found near the pial surface (Fig. 5.3 A).

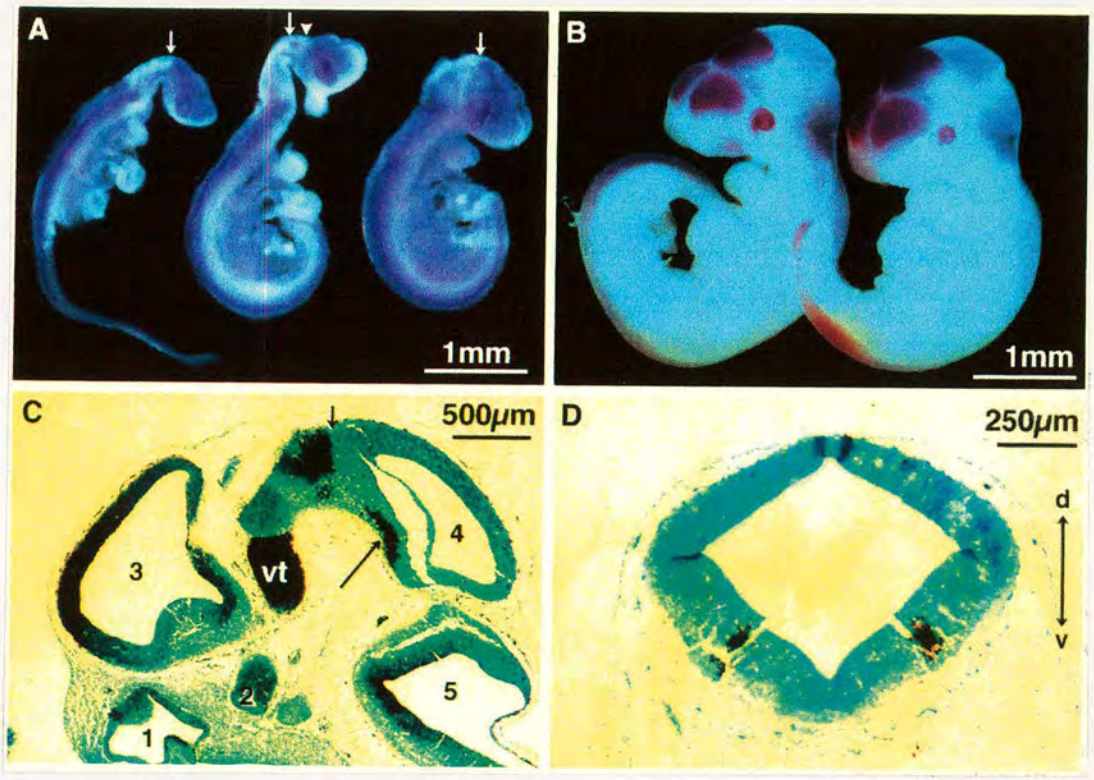

Figure 5.1
Variation in levels of *Pax6* expression along the antero-posterior and dorso-ventral axes of the developing CNS. Wholemount *in situ* hybridisation at E8.75 to E9.25(A) and at E10.5 (B) show that there is *Pax6* expresion in developing spinal chord, hindbrain and forebrain but no extensive expression in the developing midbrain. There is a clear boundary of *Pax6* expression between presumptive forebrain and midbrain (arrowed in A) even in the youngest embryo shown (left in A). Between two domains of strong *Pax6* expression in the forebrain there is a region of slighlty lower expression (arrowhead in A). (C) *Pax6* expression respects neuromere boundaries, shown at E10.5 on a parasagital section through the brain. 1, nasal cavity; 2, optic stalk; 3 lateral ventricle of telencephalon; 4 mesencephalon; 5 rhombencephalon. There is strong *Pax6* mRNA expression in the synencephalon, on the diencephalon side of the forebrain-midbrain boundary, (short arrow). *Pax6* is also strongly expressed in the presumptive ventral thalamus and there is a clear boundary in the expression between the presumptive ventral and dorsal thalamus. This section also shows the appearance of *Pax6* expression in the ventral midbrain (long arrow). (D). Narrow, bilateral bands of expression running the length of the midbrain at E11.5. The two ventrolaterally placed domains of expression do not extend to either the ventricular, or the pial, surface.

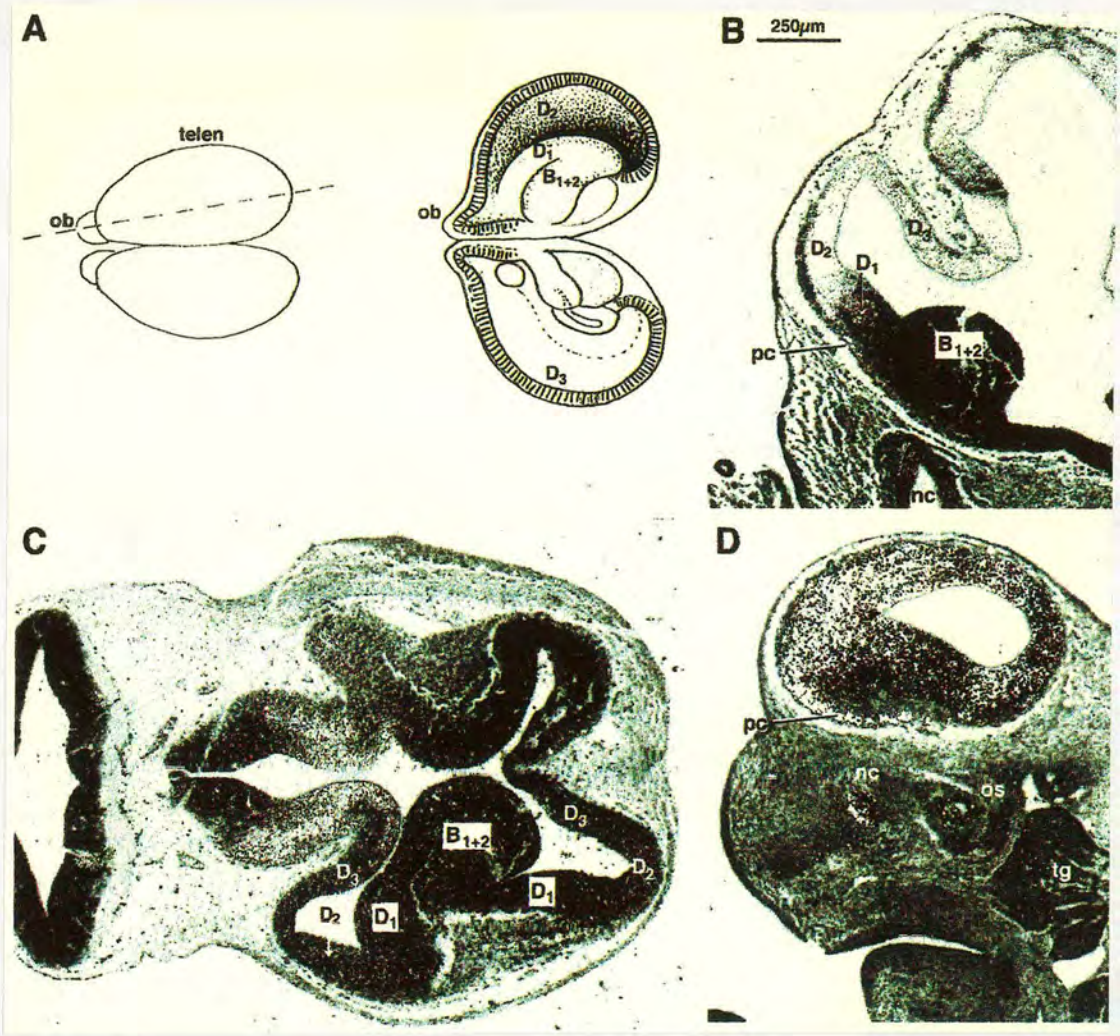

Figure 5.2.
Pax6 expression in the developing forebrain. (A) Subdivisions of the developing forebrain, based on an original figure by Ian Smart, with the notation of Kuhlenbeck (1973). A disection of the telencephalic lobes reveals lateral and medial ventricular surfaces on which dorsal (D_1, D_2 and D_3) and basal (B_{1+2}) subdivisions are indicated. (B-D) *Pax6* mRNA expression (white) overlayed on bright field images of frontal (B), transverse (C) and parasagital (D), sections at E10.5 (B and D) and E 11.5 (C). The strongest *Pax6* expression in the forebrain is in the diencephalon and the telo-diencephalic sulcus (C). (B and C) Within the telencephalon, *Pax6* is expressed most strongly in the D_2 domain with slightly weaker expression in D_3. There is some expression in D_1 (B) but the basal domains B_1 and B_2 do not express *Pax6*. (B and D) *Pax6* is strongly expressed in a band of cells, pc in the marginal ventro-lateral forebrain, in the position of the presumptive piriform cortex. Abbrieviations ob olfactory bulbs; telen, telencephalon; nc, nasal cavites; os optic stalk; tg trigeminal gangion.

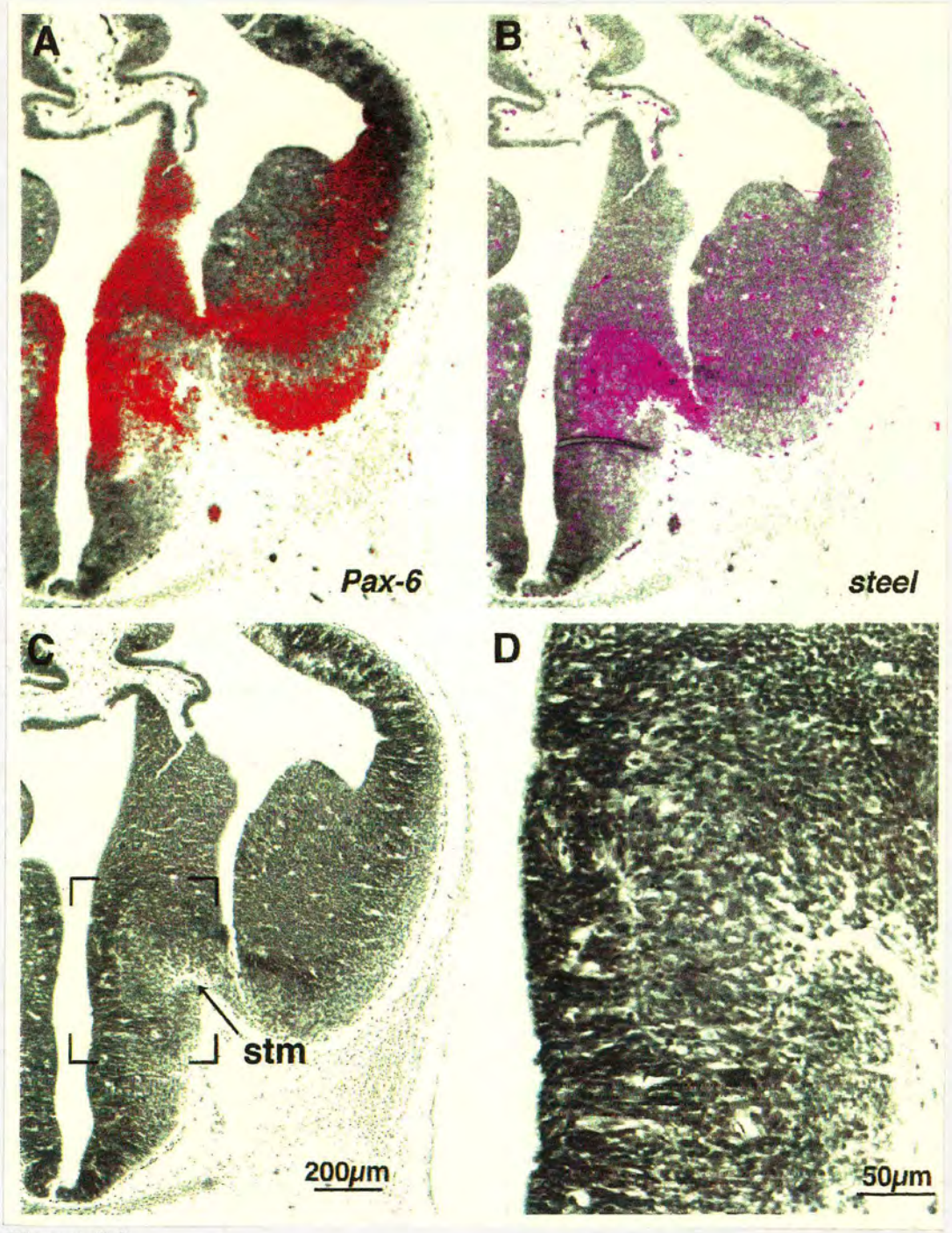

Figure 5.3.

Relationship between *Pax6* expression, *steel* expression and cell morphology at the telo-diencephalic sulcus. *Pax6* expression (A) is complementary to *steel* expression (B), found in developing stria medullaris (stm). Haemotoxylin and eosin histology of the same region (C,D) shows that *Pax6*-expressing and *steel*-expressing cell populations have distinct packing properties, with differences in cell density that respect the *Pax6 / steel* expression boundary.

5.2.3 Complementary patterns of *steel* and *Pax6* expression.

Pax6 mRNA expression in the forebrain was compared at E11.5 to the expression of other genes. *Steel*, encoding the c-kit ligand, is expressed in a pattern (Fig. 5.3 B) complementary to that of *Pax6* (Fig 5.3 A) in the telo-diencephalic sulcus region. Histological analysis (Fig 5.3 C-D) showed that in the folds of neuroepithelium, cells expressing *Pax6* have a distinct morphology from those expressing *steel*; tightly packed neuroepitheial cells express *Pax6* mRNA and more loosely packed cells forming the differentiating stria medullaris (Schambra et al, 1992) express *steel* mRNA.

5.2.4 *Pax6* expression in wild-type mice becomes increasingly spatially restricted.

At E8.75, shortly after it is first expressed in the CNS, *Pax6* expression is found in broad domains of neural ectoderm within which the majority of cells appear to be expressing the gene. By E10.5 to E11.5 small numbers of cells come to express *Pax6* at a high level within regions of lower expression. This is shown by wholemount *in situ* hybridisation in the posterior diencephalon (white arrow in Fig. 5.8A). When *Pax6* expression was examined in the more mature embryonic brains up to E17.5, strong signal using a *Pax6* probe was observed only in very limited domains of the brain as illustrated in Fig. 5.4 A, a transverse section at the level of the olfactory bulbs at E16.5. *Pax-6* probe and the control *LH-2* probe hybridise to largely non-overlapping domains. For example there is *LH-2* signal in the developing tectum but not in tegmentum (Fig. 5.4 B), whereas conversely, signal with the *Pax6*

probe is present in the tegmentum, but not in the tectum (Fig. 5.4. A). Use of such a control probe (Fig. 5.4 B and Fig. 5.4 D) thus confirms that the pattern of hybridisation obtained with a *Pax6* probe is not due to non-specific binding.

Some domains of *Pax6* expression at mid to late gestation coincide with cellular proliferation. Fig. 5.4 C shows *Pax6* expression in the developing cerebral cortex. Unlike the *LH-2* expression which is present in both germinative epithelium and developing cortical plate (Fig. 5.4 D), *Pax6* transcripts are confined to cells in the germinative epithelium at this stage. Similarly, the external granular layer of the cerebellum is a germinative epithelium and, in sections from embryos aged E15.5 to E17.5, was found to express *Pax6* mRNA (Fig. 5.4 J).

Figure 5.4. (following page)
Resticted domains of *Pax6* mRNA expression in the brain at late gestation. Transverse sections at E16.5 at level of olfactory bulbs (A-I) or pons (J). Overview of hybridisation with a *Pax6* probe (A), compared with hybridisation using the *LH-2* control probe (B). Signal with the *Pax6* probe (red) is detected in the olfactory bulbs (ob) , within the fornix (fnx), in the developing cerebral cortex (ctx), in a narrow band of cells on the thalamus side of the internal capsule (ic), and in the tegmentum (teg). (B) The largely complemetary pattern of hybridisation with the *LH-2* probe (blue) confirms the specificity of this signal. *LH-2* exprssion is present in the tectum (tc) but not tegmentum, and there is *LH-2* expression medial of the *Pax6* expression near the internal capsule. In the fornix, *LH-2* expression is medial and posterior of the *Pax6* expression domain. (C) *Pax6* expression in the developing cerebral cortex is confined to the germinative epithelium (ge), and unlike the LH-2 gene (D), *Pax6* is not expressed in the intermediate zone (iz) or developing cortical plate (cp). Olfactory bulbs express *Pax6* in the germinative epithelium (A, G) and in the glomerular cell layer (glm) and granular cell layer (grn) (E). *Pax6* is also expressed in a lateral and a medial band of cells along the paths of the lateral olfactory tract (lot) and of more medial fibers (mf) to and from the olfactory bulbs. (G, H) Detail of the relationship of *Pax6* expressing cells in the fornix to the position of the diagonal band of Broca (db). (I) Narrow band of *Pax6* expressing cells in the position of the external medullary lamina (eml), medial of the internal capsule. (J) *Pax6* expression in the germinative epithelium of the devloping cerebellum (cb) in the dorsal tegmental nucleus (dtn) and lateral lemniscus (llm).

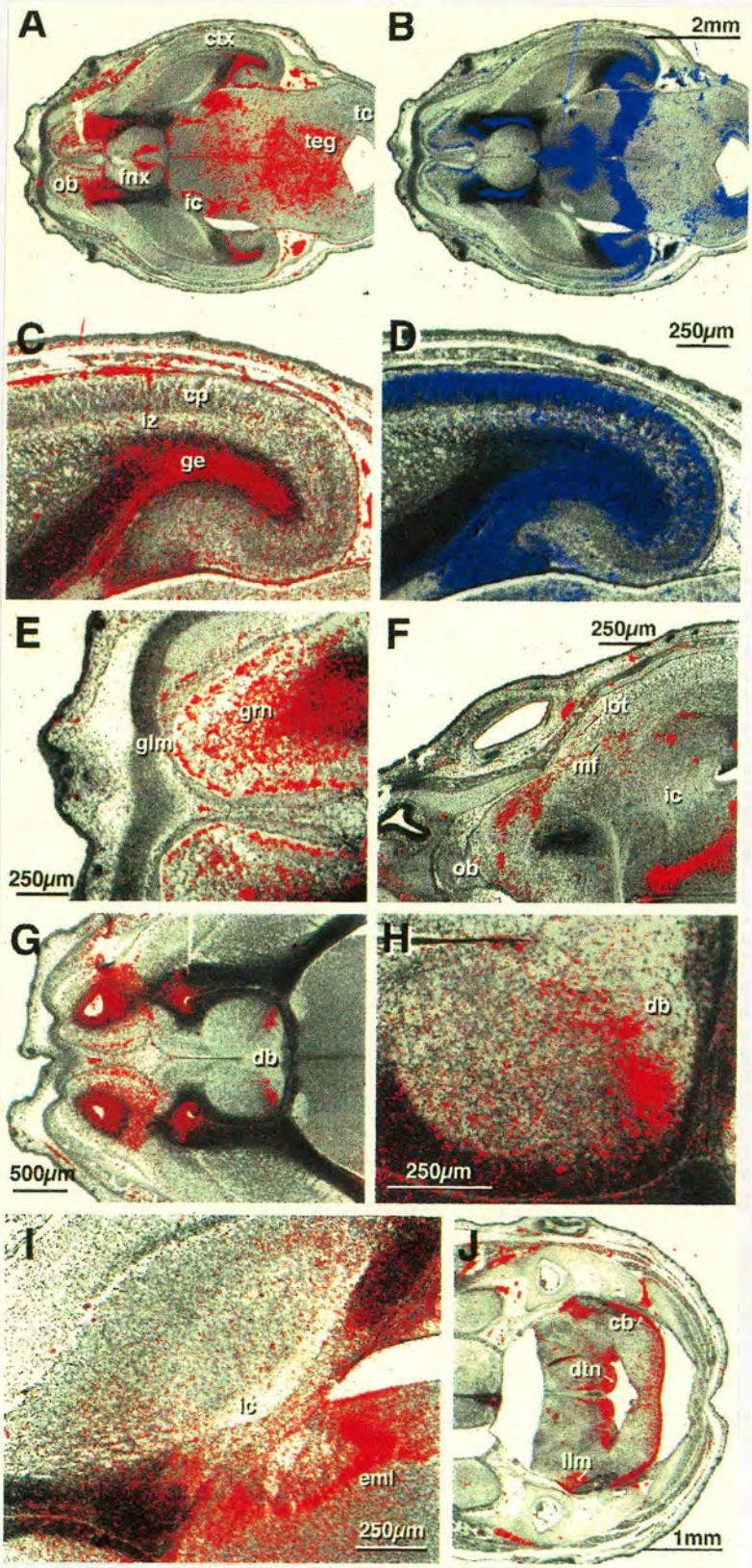

Figure 5.4 (see preceding page for legend)

5.2.5 *Pax6* expression and fibre tracts in the developing wild-type brain.

A number of the domains where *Pax6* continues to be expressed are adjacent to tracts of nerve fibres in the developing brain. In Fig. 5.4 A, *Pax6* expression is apparent in a narrow line of cells on the thalamic side of the internal capsule, in the position of the external medullary lamina (Schambra *et al.,* 1992), and there is also *Pax6* expression in a domain within the fornix, lateral to the diagonal band of Broca. The relationship of this expression to these tracts of fibres is shown in more detail in Fig. 5.4 G, Fig. 5.4 H and Fig. 5.4 I. Fibre tracts appear as lightly staining regions with few cell nuclei on these micrographs.

In the olfactory bulbs, *Pax6* is expressed in germinative epithelium and strong expression is also detected in the in the glomerular cell layer and granular cell layer (Fig. 5.4 E). The expression of the *Pax6* gene in the olfactory bulb is continuous with two bands of expression running posteriorly, one close to the at the lateral edge of the brain and one more medially (Fig. 5.4 F). These bands of expression are along the paths of the lateral olfactory tract and of more medial fibres leaving and entering the olfactory bulb.

Sections through the hindbrain at the level of the pons reveal two specific sites of *Pax6* expression, the dorsal tegemental nucleus and the lateral lemniscus (Fig. 5.4 J). The lateral lemniscus is part of the auditory system (Kahle, 1986), and so, like the tracts forming the olfactory system, is involved in the processing of sensory information.

5.3 Morphological abnormalities of the early *Sey/Sey* forebrain.

Sey/Sey mutant embryos can first be morphologically recognised at E9.25-E9.5 on the basis of forebrain abnormalities and the shape of the optic vesicle (Fig. 5.5 A,B). Such embryos were selected visually and their genotype was confirmed by PCR analysis described in the materials and methods. The optic vesicle of *Sey/Sey* embryos appears broader than normal and has failed to constrict at the proximal end. The prosencephalon lacks a clear furrow (arrowed in Fig. 5.5 A), that in normal and *Sey/+* littermates is beginning to divide the prosencephalon into telencephalon and diencephalon. These aspects of the phenotype are more readily apparent at E10-10.5, when *Sey/Sey* embryos are distinguishable by other characteristics, notably absence of lens pits and nasal pits. At this stage in normal embryos, the furrows between telencephalic lobes and between telencephalon and diencephalon are prominent (Fig 5.5 C), but in *Sey/Sey* embryos the forebrain appears swollen and the furrows are much less well developed (Fig. 5.5 D). Heads from embryos at this stage were bisected along the midline and viewed with transmitted light, in which thick or dense regions of tissue between the observer and light source appear dark (Fig. 5.5 E-F). Under these conditions, the prominent sulci between telencephalon and diencephalon and between left and right lobes of the telencephalon were readily apparent in normal and *Sey/+* embryos (Fig 5.5 E). These embryos were also seen to have proximally constricted optic vesicles. In contrast, in *Sey/Sey* embryos the boundary between diencephalon and telencephalon was only faintly detectable, and the optic vesicle were not constricted proximally and appeared to have much less tissue separating it

from the forebrain (Fig 5.5 F). From sections, it is apparent that the sulci, or folds, between telencephalon and diencephalon are initiated, but remain less prominent, in *Sey/Sey* embryos (Fig. 5.7 D).

Transmitted light was also used to view whole heads at E11.5-E12 of homozygous *Small eye* embryos and their littermates (shown in Fig. 5.5 H and Fig. 5.5 G respectively). Under these conditions, lighter regions had little tissue obscuring the light source, and there was a bright crescent in the forebrain present in *Sey/Sey* embryos, suggesting a relative paucity of tissue in this region. Overall, the telencephalon in *Sey/Sey* embryos appears to be of comparable size to that in littermates, but from sections, it appears that the telencephalon of *Sey/Sey* embryos may have abnormally thin walls (compare Fig. 5.7 E and 5.7 F). Sections also showed that the arrangement of tissues in the diencephalon /telencephalon region is abnormal. The walls of the ventral diencephalon form an acute angle at the median hinge point in littermates Fig 5.7 E, but are splayed out to a more obtuse angle in *Sey/Sey* embryos Fig 5.7 F. This abnormal separation of left and right diencephalon does not appear to become corrected at later stages. Fig. 5.5 I-J show transverse sections at E14.5 of wild type and *Sey/Sey* brains. The *Sey/Sey* brain lacks olfactory bulbs, as previously reported (Hogan et al, 1986). The third ventricle is branched in *Sey/Sey* embryos. Left and right thalamus at this level of section are closely apposed in the wild type embryo, but not in the *Sey/Sey* embryo.

Figure 5.5

Brain and optic vesicle phenotype of *Sey/Sey* embryos (right column) compared with wild-type and littermate control embryos (left column). (A, B) Wild-type (A) and *Sey/Sey* (B) embryos at E9.5. Furrow normally present in prosencephalon between presumptive diencephalon and telencephalon, shown by white arrow in (A) is absent in (B). *Sey/Sey* optic vesicle is broader than in controls and has failed to constrict proximally. Black arrow indicates proximal constriction in (A). Arrowheads delimit the extent of the eye region. (C). Morphology of forebrains of control embryos at E10. Furrows between telencephalon and diencephalon, and between left and right telencephalic lobes are arrowed. (D) *Sey/Sey* forebrain at E10 has a swollen appearance. Both the furrow between the telencephalon and diencephalon and that between left and right telencephalon, are present, but are less prominent than in controls. (E, F) Heads at E10.5 bisected along the midline. The sulci manifested as furrows on the external surface of the brain appear as thick ridges of tissue when viewed with transmitted light (arrow in E). present as furrows at E10.5, shown in (E, F), these furrows appear as thick ridges of tissue in the control embryo (E), but are much less prominent in the *Sey/Sey* embryo (F), which also has far less tissue separating the optic vesicle (ov) from the telencephalic vesicles. (G, H) Whole embryo heads at viewed with transmitted light at E12. *Sey/Sey* mutant (H) has a bright crescent (arrow) in the posterior telencephalon that is not present in the littermate control (G). With this illumination, bright regions are those with little tissue between the observer and the lightsource, thus the bright crescent suggests a relative paucity of tissue in this region. (I, J) Semi-thin plastic sections of wild-type (I) and *Sey/Sey* embryo brains at E14.5, stained with Toluidin Blue. *Sey/Sey* embryo lacks olfactory bulbs and has a branched third ventricle, with widely separated left and right thalamus, which are normally closely apposed at this level of section.

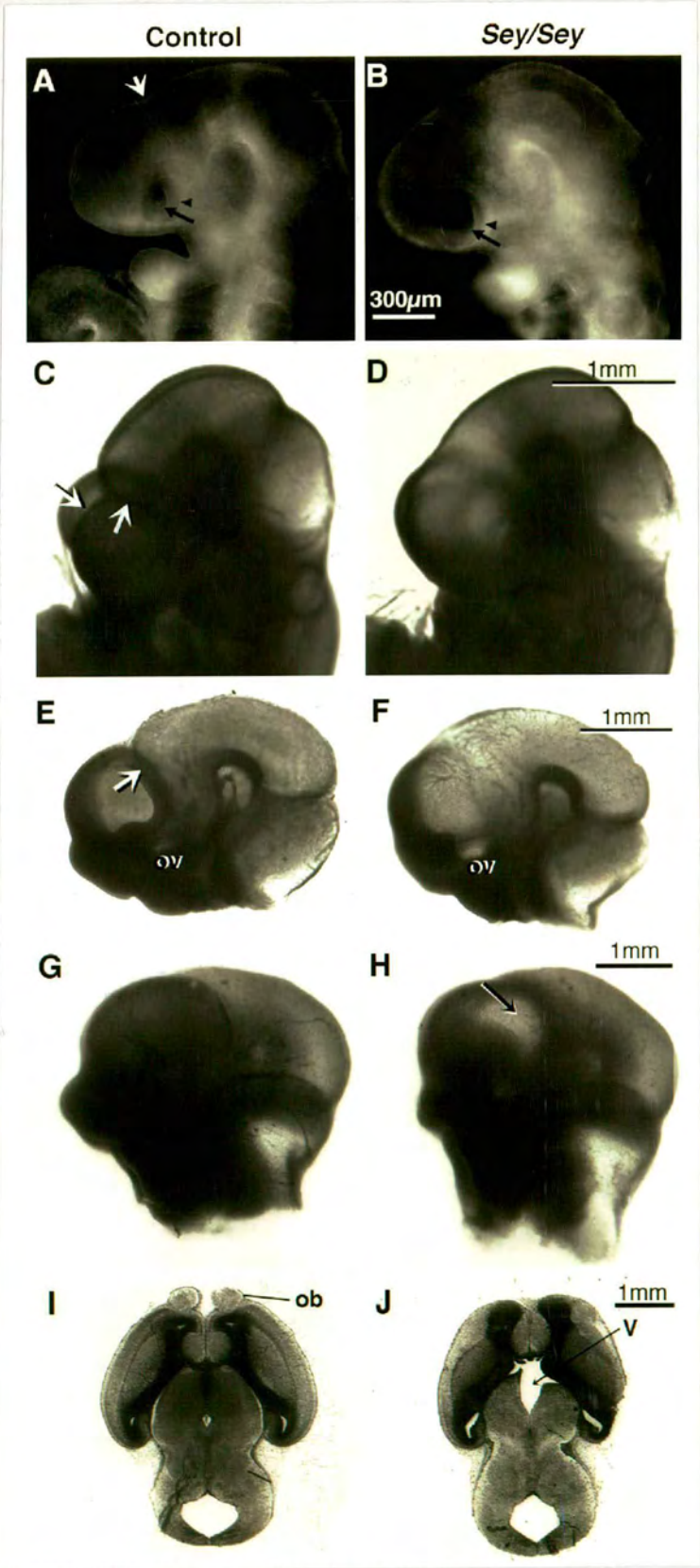

Figure 5.5 (see preceding page for legend)

5.4 Lamination defects in the developing cerebral cortex.

Schmahl et al, (1993) described defects of neuronal migration in the developing cerebral cortex of *Sey*^{Neu} mice. To provide a baseline for the comparison of these defects with the expression of *Pax6* mRNA, these defects were re-examined in mice carrying the *Sey* mutation. The normal, layered structure of the cerebral cortex at E17.5 is shown in Fig. 5.6 A. Cells produced in the germinative epithelium have migrated across the intermediate zone to form a thick cortical plate. As described by Schmahl et al, the germinative epithelium in homozygous mutants is abnormally thick and has an irregular boundary with an intermediate zone that is correspondingly reduced in thickness. The cortical plate is greatly reduced, and within the intermediate zone, there are heterotopic groups of cells with the packing density and haemotoxylin and eosin staining properties of germinative epithelium (Fig. 5.6 B). These heterotopias are particularly common in the posterior telencephalon (Fig. 5.6 C,D). One aspect of the *Sey/Sey* phenotype not reported by Schmahl et al. in *Sey*^{Neu}/*Sey*^{Neu} mice is a regular array of dense clusters of cell bodies in the dorso-lateral telencephalon (Fig. 5.6 E, F). The location of these abnormal clusters suggests that they could be in the somatosensory cortex, which normally later forms an array of barrel fields (David Price, personal communication).

Transverse sections through more basal regions of telencephalon at E14.5, show radially extending clumps of cells (Fig. 5.6 H) in place of the smooth layers of wild type embryos (Fig. 5.6 G.) This lamination defect thus slightly precedes those previously described for *Sey*^{Neu} animals (Schmahl et al, 1993).

Figure 5.6 (following page)

Lamination defects in the the developing *Sey/Sey* cerebral cortex at E17.5 (A-F), and E14.5 (G, H). (A-F) Haemotoxylin and eosin histology on transverse (A, B) and parasagital sections (C-F). (A) The normal layered structure of the developing cerebral cortex at E17.5. At this stage there is a well formed cortical plate (cp), separated from the germinative epithelium (ge) by the intermediate zone (iz), a region with many radial glial fibers, but few nuclei. (B). Abnormal cerebral cortex development in *Sey/Sey* embryos at E17.5. The cortical plate is narrower than normal. The germinative epithelium is abnormally thick and has an irregular boundary with the narrow intermediate zone which contains heterotopic clusters of cells (ht). (C and D) Heterotopic clusters of cells (ht) in the developing cortex (cx) of *Sey/Sey* animals are common near the posterior pole, shown in (D). (E and F) Parasagital sections showing dense, regularly spaced, ectopic clusters of cells of the dorso-lateral cortex of *Sey/Sey* mice. (G and H) Transverse semi-thin plastic sections stained with Toluidine Blue, through the lateral telecephalon of wild-type embryo (G), and *Sey/Sey* embryo (H), at E14.5. (G) The formation of a regular laminar structure has been initiated in wild-type embryos at E14.5. (H) *Sey/Sey* animals have defects in this lamination(ld), with clusters of strongly staining cells extending radially.

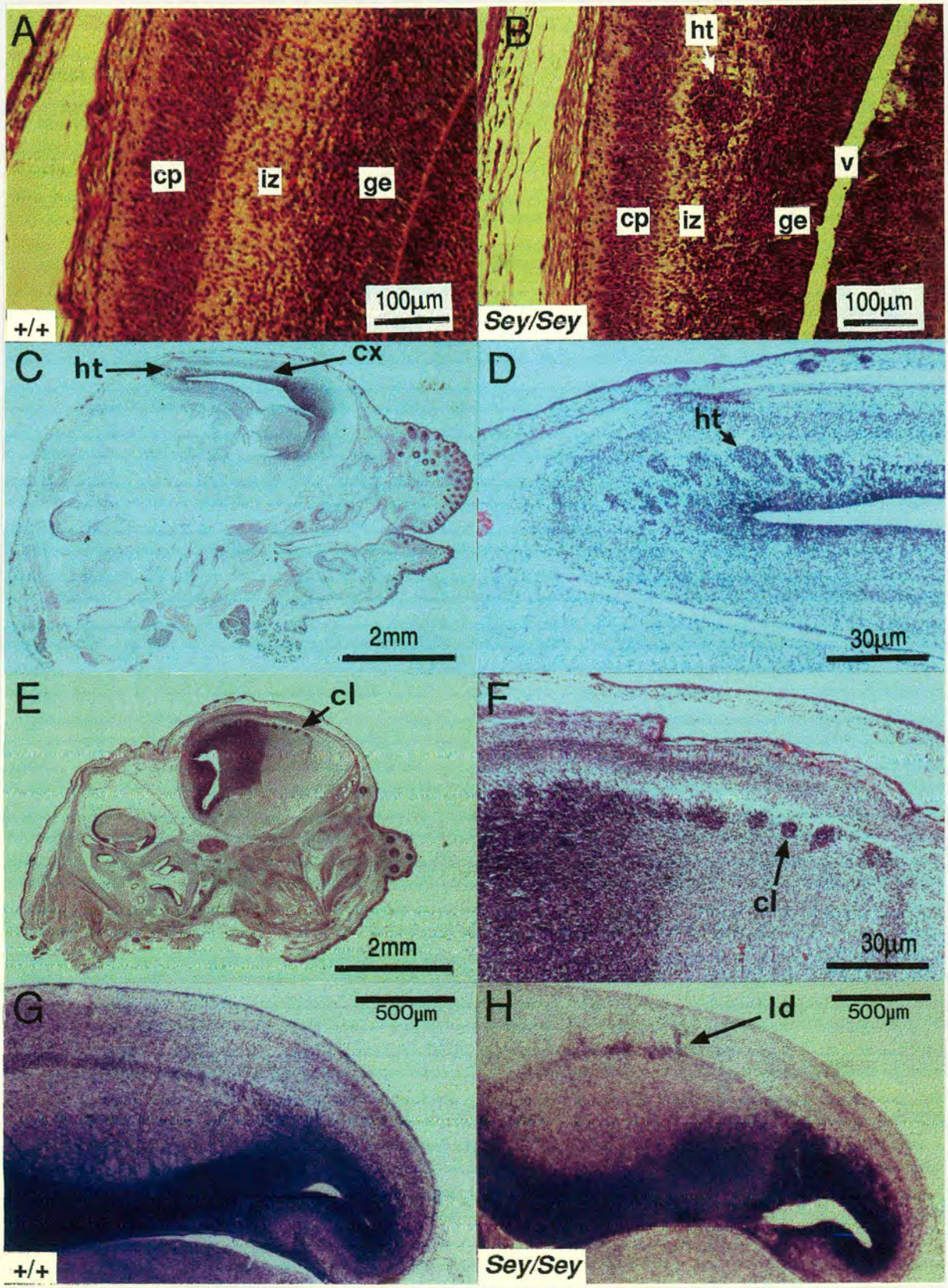

Figure 5.6 (see preceding page for legend)

5.5 Association between *Pax6* mRNA expression and *Sey/Sey* defects.

In *Sey/Sey* mutants, the proximal optic vesicle fails to constrict and the telo-diencephalic sulcus is poorly formed. Both these abnormalities are associated with sites of strong *Pax6* expression in the neural ectoderm. The single base change in the *Sey* allele (Hill et al, 1991) does not affect the *in situ* hybridisation assay for *Pax6* mRNA expression, thus we have been able to analyse *Pax6* expression in *Sey/Sey* embryos in which the relationship between phenotype and expression can be most readily assessed. The studies of expression in wild-type embryos were thus complemented by examination of *Pax6* expression in *Sey/Sey* mutants and their littermates from E9.5 to E12.5.

In general, *Pax6* transcripts in the optic vesicle are most abundant distally, particularly around the rim of the developing optic cup (Grindley et al, 1995), but , interestingly, at E9.5 there is also some strong *Pax6* mRNA expression where the neuroepithelium becomes constricted, on the dorsal-caudal side of the optic vesicle at the junction between vesicle and diencephalon (Fig 5.7 A-B).

The folding neuroepithelium at the diencephalon-telencephalon boundary is also a site of particularly strong *Pax6* mRNA expression (shown at E9.75 and E11.5 in Fig 5.7 C, and Fig 5.7 D respectively). By E11.5 (Fig. 5.7 D) the expression of *Pax6* mRNA in this fold region can be seen to be differential. There is strong *Pax6* expression in a layer a few cells thick on the ventricular surface of the fold between diencephalon and telencephalon (upper arrow in Fig. 5.7 D). Within the

diencephalon, the ventricular surface curves in a concave rather than convex manner, and here the strongest *Pax6* expression is furthest from the ventricular zone (lower arrow, Fig. 5.7 D). Examination of the levels of *Pax6* mRNA in the telencephalon-diencephalon region at earlier stages (from E9.5) revealed that expression was not uniform throughout folding neuroepithelium but rather was stronger on the convex than on the concave side (shown at E9.75 by the arrows in the detail of Fig. 5.7 C). Examination of *Pax6* expression *Sey/Sey* mutant embryos (Fig. 5.7 F and Fig. 5.7 H) shows the relationship between strong *Pax6* mRNA expression at the telencephalon-diencephalon boundary, and the failure of the fold in this region to fully form.

Figure 5.7 (see following page)

Structures affected in *Sey/Sey* mutants and their associated *Pax6* expression patterns. Frontal sections though the optic vesicles of *Sey/Sey* embryo (B) and littermate control (A) at E9.5 showing failure of *Sey/Sey* optic vesicle to constrict proximally. There is strong *Pax6* mRNA expression in neural ectoderm that forms this constriction, the dorso-caudal region of the proximal optic vesicle. Variation in levels of *Pax6* expression relates to brain topology in the telo-diencephalic sulcus (C-D). (C) Arrowheads mark telo-diencephalic sulcus in E9.75 embryo. A detail of this region is shown to the right. *Pax6* expression is strongest near to the convex surface of the folding neural ectoderm (arrows).(D) The telo-diencephalic sulcus at E11.5. *Pax6* expression is now absent from the devloping stria medullaris and is strongest close to the convex surface (arrows). Between the diencephalon and telencephalon the strongest expression is close to the ventricle, within the diencephalon it is furthest from the ventricular zone. Tranverse *in situ* sections at E10.5 (E,F) and E11.5 (G,H) show abnormal tissue organisation in *Sey/Sey* mutant forebrains (F,H) compared with littermates (E,G) with enlarged ventricular spaces and poorly developed telo-diencephalic sulci (arrows), which are sites of particularly strong *Pax6* mRNA expression. Lines in (E) and (F) show the angle at the median hinge point between left and right walls of ventral diencephalon. This angle is increased in the *Sey/Sey* mutant animal. (A,B) Brightfield histology image overlayed with inverted (black ⇔ white) darkfield *in situ* signal. (C,G,H) Brightfield images. (D,E,F) Inverted darkfield images. ov optic vesicle; dien, diencephalon; mes, mesencephalon; telen telencephalon;, stm, developing stria medullaris; mhp, median hinge point; post pole, posterior pole of telencephalon.

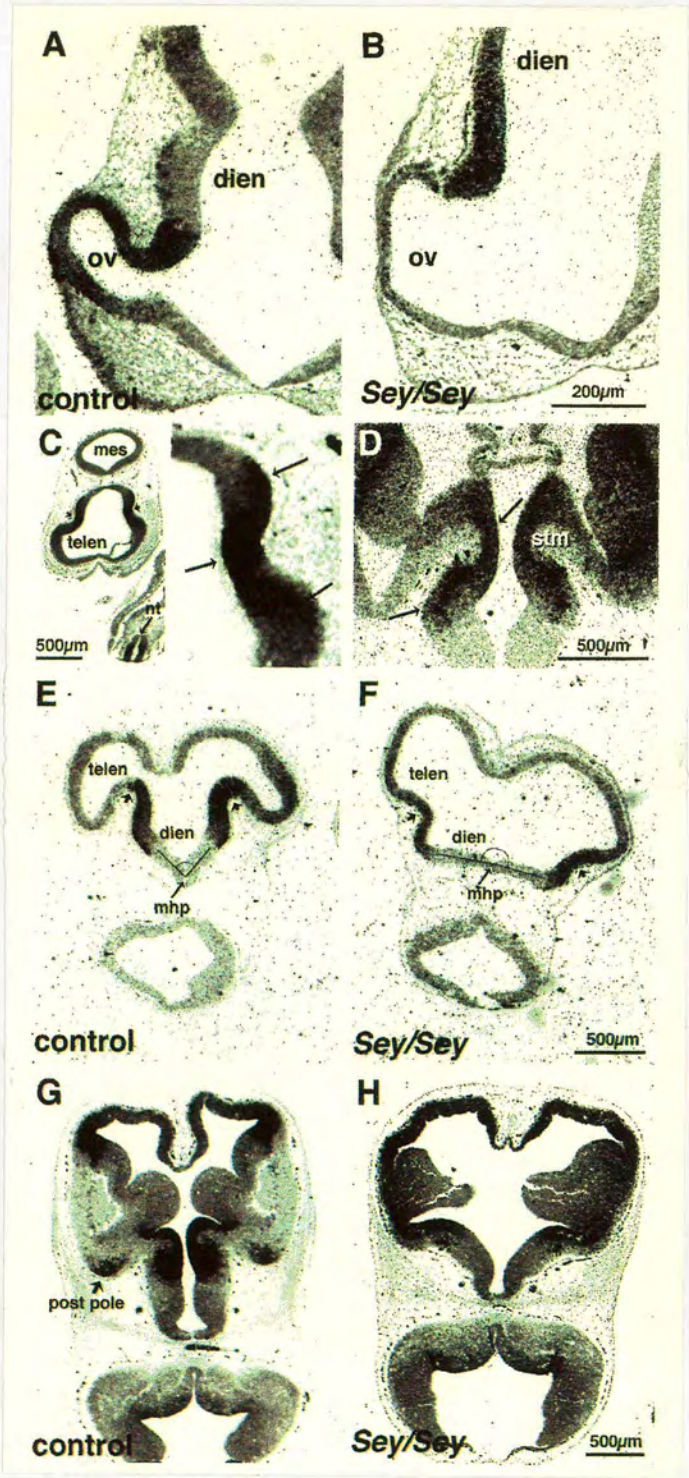

Figure 5.7 (see preceding page for legend)

5.6 *Pax6* expression at the forebrain-midbrain boundary in *Sey/Sey* mutants.

To study the relationship between *Pax6* expression and neuromere boundary formation, the formation of the *Pax6* expression boundary between forebrain and midbrain was examined in normal and *Sey/Sey* embryos at E10.5 and E11.5 by wholemount *in situ* hybridisation. At E10.5 the normal *Pax6* expression boundary is still slightly ragged and there are a few isolated expressing cells on the midbrain side of this boundary, but the boundary resolves and sharpens by E11.5. In the younger wholemounts (E 8.75-E9.25, shown in Fig. 5.1 A), the expressing field is of fairly uniform intensity, but by E10.5, bilaterally symmetrical arrangement of cells within this field of expression close to the forebrain-midbrain boundary, come to express at a high level (Fig. 5.8 A)

Fig. 5.8 B shows wholemount *in situ* hybridisation with a *Pax6* probe on a *Sey/Sey* embryo and a littermate at E11.5. The littermate exhibits the normal pattern of expression with a sharp expression boundary between forebrain and midbrain (indicated by arrows). In contrast these is no such clear boundary of *Pax6* mRNA expression in the brains of *Sey/Sey* embryos at E11.5. Instead there is strong expression more anteriorly in the diencephalon, near the prospective epiphysis, with a diffuse posterior boundary.

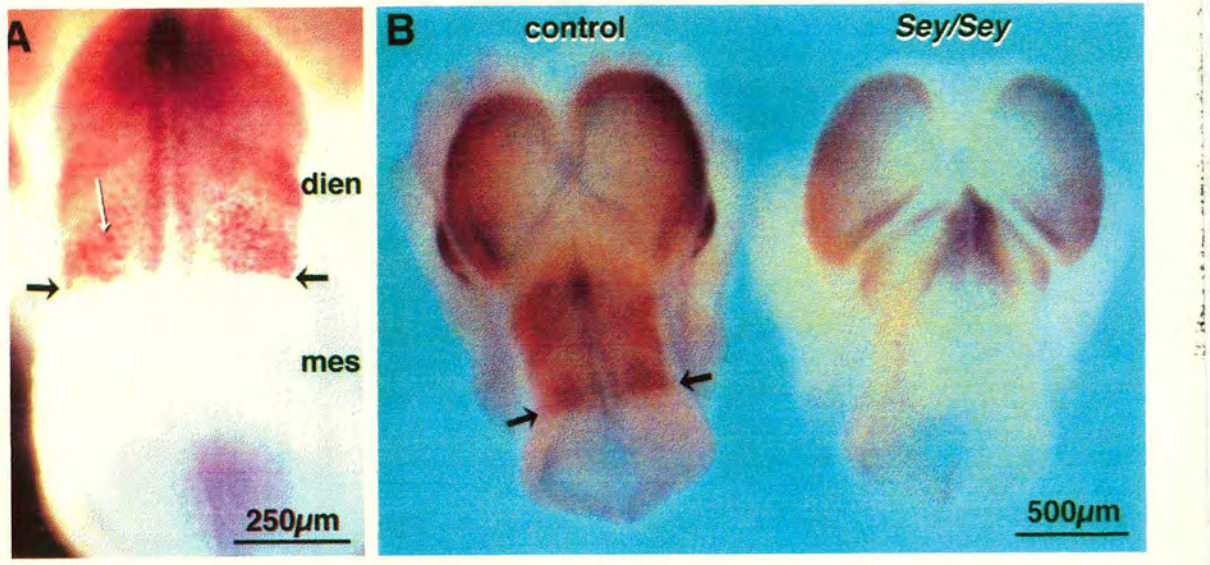

Figure 5.8

Absence of posterior diencephalon *Pax6* expression in *Sey/Sey* mutants. *Pax6* wholemount *in situ* hybridiation at E10.5 and E11.5. (A) Wild-type embryo at E10.5. Black arrows indicate boundary of *Pax6* expression between diencephalon and mesencephalon. Within the field of *Pax6* expression, some cells (white arrow) come to express *Pax6* at high levels. (B) Dorsal view of E11.5 heads of *Sey/Sey* embryo (right) and littermate (left). Clear *Pax6* expression boundary (arrowed) between developing forebrain and midbrain is present in littermate control embryo but absent from *Sey/Sey* embryo, which nevertheless retains strong *Pax6* expression more anteriorly in region of prospective epiphysis.

5.7 Morphological boundaries in the diencephalon of *Sey/Sey* embryos.

To further determine whether the morphological segment boundaries in the diencephalon were affected by *Pax6* mutations scanning electron microscopy (SEM) was performed on the inside of heads at E10.5 and E11.5. By E11.5 in control brains there are prominent ridges present (numbered one to four in Fig. 5.9 A), equivalent to those observed in the E13 rat brain by Lakke *et al.,* (1988). The first three of these sulci are approximately at neuromere boundaries, between the midbrain and forebrain, between the synencephalon and posterior parencephalon (presumptive dorsal thalamus), and between the posterior and anterior parencephalon (presumptive dorsal thalamus and presumptive ventral thalamus) (Lakke et al., 1988). The fourth of these labelled ridges (Fig. 5.9 A), between the mamillary and infundibular recesses, is the sulcus diencephalicus basalis (Lakke et al., 1988), which at this stage appears to extend dorsally to the torus hemisphericus. The location of rostral limit of the sulcus limitans, at the second of these ridges (Fig. 5.9 A), also agrees with limit found in the rat brain (Lakke et al, 1988)

Two of the sulci at neuromere boundaries are detected in the *Sey/Sey* embryos (Fig. 5.9 B). The most caudal of the recognisable sulci is at the position of the normal forebrain-midbrain boundary. The position of the next more rostral ridge suggests that it corresponds to the boundary between synencephalon and posterior parencephalon, the precursor of the dorsal thalamus. More rostrally, basal features such as the infundibular recess and mamillary recess appear normally placed, but

further segment boundaries are difficult to detect in *Sey/Sey* heads as there is typically considerable distortion of the ventricle shape in the region of the torus hemisphericus. *Sey/Sey* embryos have an abnormally broad optic vesicle associated with a less prominent preoptic area.

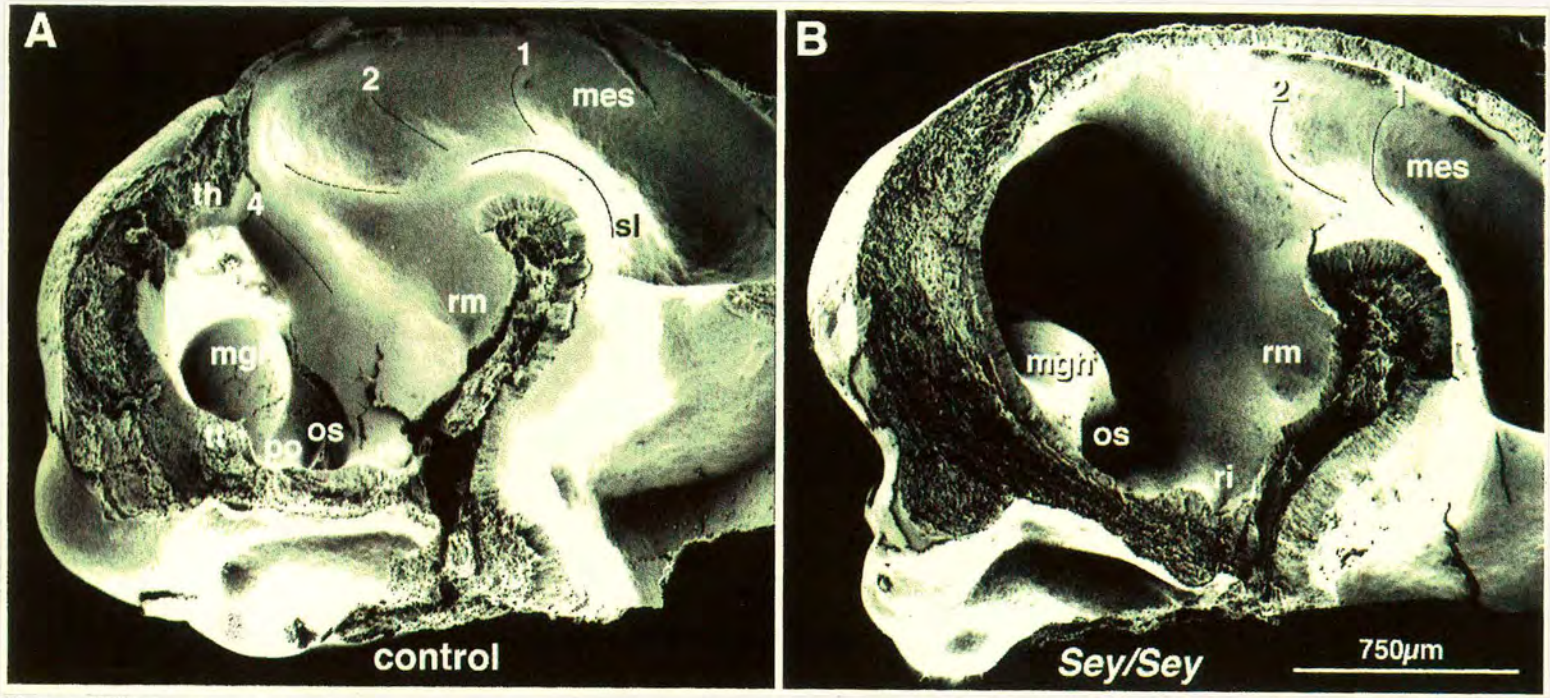

Figure 5.9

Morphological boundaries in the diencephalon. Scanning electron microscopy at E11.5 inside heads of a *Sey/Sey* embryo (B), and a littermate control embryo (A), bissected along the midline. The normal patterns of transverse sulci visible in the ventricle wall are numbered 1-4 in the control embryo (A). 1 is at the forebrain-midbrain boundary, 2 and 3 are the positions of neuromere boundaries within the diencephalon. The *Sey/Sey* embryo has the most posterior of the four sulci which marks the morphological boundary between forebrain and midbrain. More anteriorly the next such boundary is also present, but further boundaries cannot be recognised. sl, sulcus limitans; rm,mamillary recess, ri, infundibular recess; mgh median ganglionic hill; os, optic stalk; po preoptic area; tt, torus transversam; th torus hemisphericus; mes, mesencephalon.

CHAPTER 6

Discussion relating to *Pax6* expression in the CNS and to defects in brain development in *Small eye* mice

6.1 Context of this study

In vertebrate brain development, elaborate structures with diverse cellular identities must be produced from a simple sheet of cells, the neural plate. A variety of morphological changes are involved in this process, with evaginations, folding and growth all contributing to the shape and organisation of the early brain. Moreover regional identity must be established, such that the different areas of the brain differentiate into morphologically and functionally distinct structures. Development of the brain is also intimately related to facial development. Thus, molecular insight into early brain development can be vital in understanding not only later brain organisation, but also a whole range of developmental processes and interactions.

Although there are now many genes known whose patterns of expression suggest roles in early forebrain development, only in a few cases has such an involvement been demonstrated. There is expression during early forebrain development of transcription factor genes of the *Pax, Dlx, Emx, Otx and HNF3* families, the *wingless* related genes, *Wnt-3* and *Wnt-3a*, and the *Tlx* genes related to the *tailless* nuclear receptor (reviewed by Puelles and Rubenstein, 1993; additional references: Tao and Lai, 1992; Monaghan *et al.,* 1993; Sasaki and Hogan , 1993; Stoykova and Gruss, 1994; Takada *et al.,* 1994; Yu *et al.,* 1994). The results of mutations in most of the genes listed above have yet to be described, but the mutant phenotypes that have been reported suggest that a forebrain expression pattern is not always a good indicator of the importance of a gene in early forebrain development. *HNF-3* β deficient mice (Weinstein *et al.*, 1994; Ang and Rossant, 1994) have no

node, notochord and floor plate, with severe consequences for dorsal ventral patterning of the nervous system and development of anterior neural structures. In contrast, a *Wnt-3a* mutation has shown the importance of this gene in caudal somite, notochord and tailbud formation, but forebrain development was apparently unaffected in these mice (Takada *et al.,* 1994).

Some genes important in early forebrain development have, however, been identified from the analysis of human dysmorphic syndromes and classical mouse mutants. These include the *KAL* gene, encoding a protein with some structural features of cell and substrate adhesion molecules (Franco *et al.*, 1991; Legouis *et al.,* 1991), and two genes, *Gli3* and *Pax6,* both encoding transcription factors.

X-linked Kallmann syndrome, in which the olfactory bulbs fail to develop, results from defects in the *KAL* gene (Franco *et al.*, 1991; Legouis *et al.,* 1991). In Kallmann syndrome patients, the olfactory epithelium develops normally, but olfactory axons fail to enter the telencephalon, and neurons from the olfactory epithelium, expressing gonadotropin-hormone-releasing hormone (GnRH), fail to complete their migration into the telencephalon (Schwanzel-Fukada *et al.,* 1989). *KAL* gene expression (Lutz *et al.,* 1994) suggests that KAL is important within the telencephalon for the inward migration of axons and neurons. The *GLI3* gene, encoding a zinc-finger transcription factor, is disrupted both in human Greig cephalopolysydactaly (Vortkamp *et al.,* 1991) and in *Extra-toe* (Vortkamp *et al.,* 1992) a mouse mutant with abnormal forebrain development, including absence of olfactory bulbs in homozygous mutants (Franz, 1994).

The importance of *Pax6* in forebrain development is indicated by the absence of the olfactory bulbs in homozygous mutant embryos of both mouse and rat *Small eye* (Hogan *et al.,* 1986; Matsuo *et al.,* 1993) and further supported by the finding that *Small eye* mice also have defects in neuronal migration and differentiation detectable in the developing cerebral cortex at late gestation (Schmahl *et al.,* 1993). More recently an anopthalmic child, a compound heterozygote for two *PAX6* mutations, was found to have brain abnormalities similar to those seen in *Sey/Sey* fetuses (Glaser *et al.,* 1994).

Since the lack of olfactory bulbs could be a consequence of failed nasal development, it is necessary to look beyond the absence of olfactory bulbs to other phenotypes of *Sey/Sey* embryos, to identify a role for *Pax6* within the brain, bearing in mind that later phenotypes might be themselves be partly due to the failure of olfactory bulb formation (Naruse and Keino, 1993). In this study an early forebrain shape defect in *Sey/Sey* embryos is described, that is not be so readily explained in terms of failed nasal development. *Pax6* mRNA expression was detected in the forebrain (this study; Walther and Gruss, 1991) around the time these defects appear. Moreover the most affected structures in *Sey/Sey* embryos are normally sites of strong *Pax6* mRNA expression. From these results it is likely that there is a direct for *Pax6* within the early forebrain.

The complex patterns of *Pax6* mRNA expression can reveal the extent of patterning within the early CNS. In the sections that follow, the information gained from the *Pax6* expression studies is reviewed along with the roles for the *Pax6* gene suggested by the expression patterns. Aspects of the *Sey/Sey* phenotype attributable

to different underlying defects are then discussed, starting with the possible phenotypic consequences of a lack of the nasal placodes. Aberrant neural crest migration, hydrocephaly, direct involvement of *Pax6* in neuroepithelium folding, and disrupted antero-posterior patterning of the diencephalon are all also raised as possible explanations for early forebrain abnormalities of *Sey/Sey* mice.

6.2 *Pax6* mRNA expression provides information on CNS patterning

Pax6 expression defines domains within the developing brain prior to, and coincident with, the times that morphological differences between brain regions become apparent. For recognised subdivisions of the brain, it is of interest to know at what stages differences between domains are evident at a molecular level, and how these differences relate to the commitment of cells within them to a region-specific fate. For example, at the times when there is differential *Pax-6* expression within the telencephalon, region-specific fates are being acquired there, such as whether or not to express the limbic system marker, LAMP (Barbe and Levitt, 1991).

Pax6 expression also reveals novel patterning within the brain, such as the narrow bi-lateral bands of expression along the length of the ventrolateral midbrain. In the case of *Pax6* expression in the telo-diencephalic sulcus, the fact that another gene, *steel*, shares an expression boundary with *Pax6*, suggests that this could be a significant boundary within the developing embryo. The ventral limit of *Pax-6* expression within the diencephalon may also reveal an important boundary within the brain. In most of the diencephalon there is a 'step' in this boundary such that the *Pax-*

6 expression extends more ventrally in the mantle layer than in the germinative layer of the neural tube. Other genes, such as *Dlx-1* and *Dbx* and *HNF3α,* (Price *et al.,* 1991; Lu *et al.,* 1992; Salinas and Nusse, 1992, Monaghan *et al.,* 1993) also have an offset between their domains of expression in the mantle and ventricular layers, suggesting that the staggered dorso-ventral boundaries may be an important feature of brain organisation.

6.3 *Pax6* and the proliferation/migration transition.

In a number of locations within the brain, including cerebral cortex, olfactory bulbs and cerebellum, there is strong *Pax6* expression in the germinative epithelium. In the cerebral cortex it is apparent that cells that have migrated away from this zone do not express *Pax6*. The work of Schmahl *et al.* (1993), repeated here using the *Sey* allele of *Small eye*, shows that homozygous *Small eye* mice have an enlargement of the germinative epithelium, and heterotopic clusters of cells are found away from the ventricular zone with the characteristics of germinative epithelium. Schmahl *et al* (1993) suggested that the differentiation of neurons in homozygous *Small eye* embryos is arrested after their final mitosis of neurons but prior to their migration. They interpreted the milder defects of heterozygous mutant animals as cells being delayed but blocked at the same point (Schmahl *et al.,* 1993; Wolfgang Schmahl, personal communication). Interestingly, the transition from proliferation to migration is also marked by a change in *Pax6* expression, as migrating cells in the intermediate zone do not express *Pax6*. Changing *Pax6* expression could be important for this

transition process, just as downregulation of *Pax2* appears to be important in kidney development (Dressler *et al.,* 1993).

6.4 *Pax6* expression and the developing olfactory system.

Pax6 expression has been described in the nasal placode (Grindley *et al.,* 1995) and subsequently in the olfactory epithelium (Walther and Gruss, 1991) as well as in the developing olfactory bulb (Walther and Gruss, 1991). These *Pax6* expressing components of the olfactory system fail to form in *Sey/Sey* mice (Hogan *et al.,* 1986, Grindley *et al.*, 1995). The strong *Pax6* expression in the telo-diencephalic sulcus is adjacent to the region that forms the stria medullaris. One of the functions of this tract of fibres is in the olfactory system. There is also *Pax6* expression in the ventro-lateral forebrain domains forming the piriform cortex, thought to process olfactory information (Valverde, 1965), and adjacent to the lateral and medial olfactory tracts. Thus other regions of *Pax6* expression may also relate to the structures important for processing olfactory information.

Olfaction is important for many aspects of behaviour. In many mammals it is not only used in feeding but is also important in sexual behaviour, social organisation, detecting predators and navigation. The phylogenetically older parts of the cerebral hemispheres, to which the olfactory fibres project, form part of the limbic system. These areas are often not principally concerned with processing olfactory information, but instead are considered to be important in emotional behaviour (Kahle, 1986). *Pax6* expression has been associated with the limbic

system, (Stoykova and Gruss, 1994) in agreement with the association with the olfactory system, found here.

There are specific domains of *Pax6* expression adjacent to certain axon tracts, one common feature of which might be in the transmission of olfactory information. In agreement with the present work, a relationship between the domains of *Pax6* expression (Krauss *et al.,* 1991), and the scaffold of axon tracts (Wilson *et al.,* 1990), has been reported for zebrafish (Macdonald *et al.*, 1994). How axon tracts come to lie along the boundaries of *Pax6* expression could also have some bearing on the early brain shape phenotype. If, for example, PAX6 regulates the expression of some cell surface or extracellular matrix molecules recognised by growth cones, then altered expression of these could affect brain morphology .

6.5 Multiple functions for *Pax6* in head development

Pax6 expression alone suggests some roles for *Pax6* within the brain. Both the expression of *Pax6* during development in head development and the pattern of defects resulting from *Pax6* mutations are complex, however. The lens, iris, cornea, optic vesicle, nasal cavities, olfactory bulbs, cerebral cortex and cerebellum all express *Pax6* mRNA during their development (Walther and Gruss, 1991; Grindley *et al.,* 1995). Analysis of the defects in aniridia, Peter's anomaly and mouse and rat *Small eye* indicates that all these tissues develop abnormally when there are mutations either in a single allele of *Pax6* (Jordan *et al.,* 1992; Hanson *et al.,* 1994; Schmahl *et al*; 1993) or in both *Pax6* alleles (Hogan *et al.,* 1986; Hill *et al.,* 1991; Matsuo *et al.,* 1993, Schmahl et al; 1993; Grindley *et al.,* 1995). *Pax6* may have

multiple roles in head development which are difficult to separate because the gene may function in interacting tissues.

The early forebrain defects of *Sey/Sey* embryos are example of the difficulty separating is difficulty, since these embryos also have severe defects in eye and nasal development (Hogan *et al.,* 1986; Matsuo *et al.,* 1993, Grindley *et al.,* 1995), and it is likely that the brain abnormalities stem in part from abnormalities of nasal development.

6.6 Nasal placodes and forebrain development.

The intimate relationship between brain and facial development can be seen in the co-incidence of brain abnormalities and facial dysgenesis (DeMeyer, 1975, Juriloff *et al.,* 1985), the neural crest origin of many facial structures (reviewed by Hörstadius 1950; Osumi-Yamashita *et al.,* 1994), and changes in mitotic activity that occur within the brain upon surgical removal of developing sense organs such as the eye (Kollros, 1982)

The developing nose has a considerable impact on the development of the brain. There are many possible reasons for this. 1) Olfactory axons from the nasal placodes probably induce the formation of the olfactory bulbs, so these structures will not be present in the absence of olfactory nerves (Gong and Shipley, 1995). 2) The absence of olfactory bulbs itself has impact on the development of the remainder of the brain (Naruse and Keino, 1993). 3) The nasal placode is also a source of hormone-producing cell populations, which enter the brain and may influence its development (Schwanzel-Fukuda and Pfaff, 1989, 1990). 4) The nose is a source of

developmentally important diffusible molecules which may influence the development of the brain directly (Crossley and Martin, 1995). 5) The mesenchyme underlying the nose may be affected by nasal development and it turn influence the subjacent brain (LaMantia *et al.,* 1993).

In the following sections, the various ways in nasal development could affect the development of the brain will be considered. The *Sey/Sey* phenotype includes an early brain shape defect, absence of olfactory bulbs and later defects of axon tract formation (This study; Hogan *et al.,* 1986; Schmahl *et al.,* 1993). All these phenotypes may partly result from defects of nasal development, but each may also reflect a requirement for PAX6 function within the brain.

6.6.1 Forebrain shape defects and absence of nasal placodes.

This study has identified defects early in the development of the forebrain in *Sey/Sey* embryos, manifested by the swollen appearance of the prosencephalon at E9.5, failure of the normal proximal constriction of the optic vesicle and failure of the prosencephalon to become fully partitioned into telencephalic vesicles and diencephalon. These defects may lead to the abnormal ventricle pattern described in late prenatal mouse embryos (Schmahl *et al.,* 1993), and in a human compound heterozygote carrying two *PAX6* mutations (Glaser *et al.,* 1994).

A feature of the *Sey/Sey* phenotype is the enlarged, misshapen ventricles. Similar effects on ventricle shape are a consistent finding of studies in which nasal placodes are manipulated or removed. In the pioneering experiments of Burr (1916), removal of one or both nasal placodes of *Amblystoma* embryos produced a marked reduction in the size of the telencephalon on the operated side. In these studies, Burr

found a progressive enlargement of brain forebrain ventricles from normal to unilateral and bilateral ablations of the nasal placode. In the study by Clairambault (1968), the youngest embryos from which placodes were removed developed large supernumerary ventricular spaces in the forebrain. Graziadei and Monti-Graziadei, (1992) found that partitioning of the forebrain into left and right hemispheres was disrupted by surgical removal of the *Xenopus* olfactory placodes. Also using *Xenopus*, Byrd and Burd, (1993) examined the more prominent olfactory bulbs that can result upon grafting an additional nasal placode. They found that the bulbs were of normal size, but had ventricles that extended more anteriorly.

The mechanism by which nasal placode manipulations affect ventricle shape are unclear. Characterisation of these telencephalon size reductions by Harrison (1958) and by Clairambault (1968) found that several different factors contributed to this aspect of the phenotype. Specifically, there was a reduced rate of cell production, enhanced cell death and also a reduction in the size of cells in some regions. These factors may well contribute to the ventricle size and shape as well as to the overall size of the telencephalon.

The effects of unilateral placode removal are widespread and extend to the contralateral hemisphere, in which mitotic rates are also reduced, and to secondary and tertiary tracts of the olfactory system, which are drastically reduced or absent (Clairambault, 1968). The nasal placodes thus influence distant parts of the brain, which may be due to a dependence on nasal placodes in the development of intermediate structures, such as the olfactory bulbs.

6.6.2 Induction of olfactory bulbs

In *Sey/Sey* embryos the absence of olfactory bulbs could be a secondary consequence of failure to produce nasal placodes and olfactory nerves. Current evidence suggests that formation of olfactory bulbs is triggered by the olfactory nerve penetrating the forebrain. 1) Ectopic olfactory nerves produced by nasal placode grafts, can enter the brain, affect local proliferation and in some cases induce the formation of ectopic glomeruli, structures normally found in the olfactory bulb (Bell 1906, 1907; Stout and Graziadei, 1980). 2) The relative timing of changes in the forebrain and olfactory nerve penetration in rat (Gong and Shipley, 1995) and mouse (Hinds 1972a,b; Doucette, 1989) are consistent with the idea that olfactory nerve penetration induces olfactory bulb formation. 3) *Polydactyly nagoya* (*Pdn*) mutant mice (*Pdn/Pdn*) mice fail to develop olfactory bulbs. These mice have nasal placodes that produce nasal cavities and olfactory axon fibres are present, but the fibres do not penetrate the brain (Naruse and Keino, 1993). Forming an olfactory nerve involves both axon growth and the de-lamination of Schwann cells from the nasal placodes from the nasal placodes. Thus, lack of nasal placodes would be sufficient to explain the absence of olfactory bulbs in *Sey/Sey* embryos.

Pax6 mRNA is expressed in the developing forebrain prior to the formation of olfactory bulbs and in the developing bulbs themselves. Thus, a direct role for the *Pax6* gene in olfactory bulb development is also a possibility. Such a role would be masked in the *Sey/Sey* mutant however, since the lack of nasal placodes would also cause a failure of olfactory bulb formation. Thus, to identify a role of the *Pax6* gene

associated with expression within the brain, it is necessary to look beyond the absence of olfactory bulbs at other aspects of the *Sey/Sey* phenotype.

6.6.3 Influence of the olfactory bulbs on the brain.

With extensive fiber tracts from the olfactory bulbs to other regions of the brain, some effect on these regions is to be expected if olfactory bulbs fail to form. Arhinencephaly, absence of olfactory bulbs, has commonly been described as part of an undivided forebrain (see Kuhlenbeck and Globus, 1936). Arhinencephaly can however also be associated with a number of other, more subtle, brain abnormalities (Kobori *et al.,* 1987). These include cortical malformations, cerebellar hypoplasia, and defects in the optic pathway. One relatively common finding is agenesis of the corpus callosum. Naruse and Keino, (1993) examined the effect of laser ablation of olfactory bulbs and found that the corpus callosum fails to cross midline. Furthermore they suggest that an enlargement of cortex may have occurred to replace the olfactory bulb region. It is thus likely that some defects in arhinecephalic brains reflect the importance of olfactory bulb development for the rest of the brain.

Some of the neuropathology of *Sey/Sey* embryos (Schmahl *et al.,* 1993) may be due to lack of olfactory bulbs. There are interesting differences however between the *Sey/Sey* phenotype and the *Polydactyly nagoya* or laser ablated animals. In both the latter cases the corpus callosum fibres are formed, but are unable to cross the midline (Naruse and Keino, 1993), yet in *Sey/Sey* embryos no corpus callosum fibres were detected (Anthony LaMantia, personal communication). Thus, this aspect of the phenotype is not merely a consequence of failed olfactory bulb formation, but probably reflects a requirement for *Pax6* gene function within the brain.

6.6.4 Forebrain defects preceding olfactory bulb induction.

Absence of olfactory bulbs can follow from a failure to produce placode derived cell types that form the olfactory nerve. The early forebrain defects in *Sey/Sey* embryos are, however, unlikely to be due to a failure of placode derived cell populations to enter the brain. In the mouse, axon outgrowth is only detected once there is a well-formed nasal pit, and this outgrowth precedes the appearance of Schwaan cells that accompany the olfactory neurons (Cuschieri and Bannister, 1975). By contrast *Sey/Sey* brain shape defects are detectable some 24 hours earlier.

Hormone-producing cell types migrate from the nasal placodes into the brain and could influence its development (Jennes, 1989; Schwanzel-Fukuda and Pfaff, 1989; Daikoku-Ishido *et al.,* 1990; Schwanzel-Fukuda and Pfaff, 1990; Livne *et al.,* 1992.; Zheng *et al.,* 1992; Murakami and Arai, 1994). The known and expected effects of loss of these hormones are in late gestation or postnatal development, and moreover, the cells producing them acquire a secretory phenotype too late to be involved in the early forebrain defects of *Sey/Sey* mice (Daikoku-Ishido *et al.,* 1990; Zheng *et al.,* 1992)

For an explanation for the early *Sey/Sey* defects, two other types of mechanism must be considered. Firstly, ways in which nasal development might influence brain development without placode-derived cells entering the brain. Secondly, mechanisms that do not involve an influence of the nose on the brain.

6.6.5 The nose as a source of diffusible molecules.

It is possible that the nasal placodes could exert an influence on brain development by mechanisms other than by producing cells that penetrate the brain. For example the nasal region may be a source of diffusible molecules that could affect brain development at a distance. A possible example would be FGF8, which is expressed by the nasal placode (Crossley and Martin, 1995). FGF8 and some FGF receptors are also expressed in the developing forebrain (Peters *et al.,* 1992,1993; Freisel *et al.,* 1992; Crossley and Martin, 1995), so the brain may be responsive to such a molecule. Intervening tissues might limit such a signal, but could also mediate a signal from placode to brain. Moreover the mesenchyme could itself be the source of factors influencing brain development.

6.7 Possible relationships of neural crest cell migration defects to *Pax6* expression.

Neural crest cell derived migrate from both the forebrain and the midbrain into the developing nasal processes (Osumi-Yamashita *et al.,* 1994). In *rSey/rSey* embryos, midbrain neural crest cells fail to migrate into the nasal region. (Matsuo *et al.*, 1993). Absence of these cells from the nasal region, or their ectopic presence elsewhere in the head, might explain the early forebrain defects. At the stages examined in the current study, there is expression of *Pax6* mRNA in the dorsal presumptive prosencephalon, thus mutations in *Pax6* might influence forebrain neural crest cells prior to their emigration. No evidence was found of *Pax6* mRNA expression in dorsal midbrain however, or in any migrating neural crest, thus the

aberrant neural crest cell migration identified by Matsuo *et al.*, (1993) is probably a secondary defect.

How might *Pax6* mutations result in neural crest migration abnormalities? Over the time-scale of this migration in the mouse (Osumi-Yamashita *et al.,* 1994) there is *Pax6* mRNA expression (Grindley *et al.,* 1995) in the developing nasal placode, a possible target for these cells, and in head surface ectoderm and neural ectoderm (Walther *et al.,* 1991; Grindley *et al.,* 1995) flanking the migration pathways (Serbedzija *et al.,* 1992). Failure of the nasal placode region to attract the migrating cells, or an abnormal environment along the migration pathway might explain how *Pax6* mutations result in neural crest cell migration defects.

One possible influence of nasal region neural crest cells on forebrain development is through retinoid signalling. Mesenchymal-like cells subjacent to the nasal placode and possibly of neural crest origin (Osumi-Yamashita *et al.,* 1994), produce retinoic acid (RA) (LaMantia *et al.*, 1993). Moreover, ventro-lateral forebrain is a target of RA-mediated gene activation (LaMantia *et al.*, 1993) and treatment with retinoids is known to produce defects in forebrain development (Durston *et al.,* 1989; Agarwal and Sato, 1993; Zimmer and Zimmer, 1992).

Whatever the origin and consequences of abnormal neural crest migration, it is clear that secondary defects in non-expressing tissues do occur in *Sey/Sey* embryos and thus must be considered in any attempt to explain the mutant phenotype.

6.8 Hydrocephaly in *Sey/Sey* forebrain defects

The early *Sey/Sey* phenotype includes internal hydrocephaly. This may be a secondary consequence of defects in brain shape or the cause of these defects. A positive cerebrospinal fluid pressure normally develops with the occlusion of the spinal canal and is required for the expansion of the forebrain.(Pexieder and Jelinek, 1970; Desmond and Jacobson, 1977). Abnormally high cerebrospinal fluid pressure might overexpand the brain and give the swollen forebrain phenotype seen in *Sey/Sey* embryos. Such a mechanism can only operate once both anterior neuropore closure and occlusion of the spinal canal have occurred, creating a closed fluid compartment in the brain ventricles and rostral spinal canal. Both these events normally occur by E9.5 in the mouse (Kaufman, 1983; Theiler, 1989; Kaufman, 1992) about the time that *Sey/Sey* brain defects become apparent. Abnormally high cerebrospinal fluid pressure may thus contribute to the brain abnormalities observed, but might also be expected to affect the whole brain, and such defects have not been identified in the development of the midbrain or hindbrain.

6.9 *Pax6* mRNA expression in affected structures.

Formation of the telo-diencephalic sulcus is severely affected in *Sey/Sey* embryos. Interestingly, in normal mice, the neural ectoderm of this sulcus is a site of strong *Pax6* mRNA expression when the sulcus is forming. Another noticeable abnormality of *Sey/Sey* embryos is the failure of the proximal optic vesicle to constrict. Whilst *Pax6* expression is generally strong in distal optic vesicle and

weaker proximally, there is some strong *Pax6* mRNA expression in the tissue normally involved in the constriction.

In general, forming a fold in a tissue probably involves co-ordinated cell behaviour. The particular combination of properties involved in producing a fold might vary from tissue to tissue, but could include such things as patterns of cell division, movement or death, the shape or adhesive properties of cells or properties of basement membranes (for reviews see Morriss-Kay, 1981; Jacobson, 1981; Schoenwolf and Smith, 1990). It is unlikely that *Pax6* expression provides the initial cue to the neural ectoderm to form folds and constrictions, since these do form in *Sey/Sey* embryos but are less prominent. Instead I suggest that loss of functional Pax6 affects some behaviour of cells in the region of neural ectoderm in which *Pax6* it is expressed, resulting in altered mechanics of fold formation. The forebrain domains of *Pax6* mRNA expression are broad compared with the extent of the telo-diencephalic sulcus, extending both rostrally and caudally of the folded region. This pattern also argues that *Pax6* expression does not provide an instruction to fold at a precise location, but might, over a broader domain of neural ectoderm, govern properties that enhance the folding process.

If differences in cell behaviour exist between one side of a tissue and the other, these could in principle contribute to the normal changes in brain morphology. Whilst the *Pax6* expression patterns in the telo-diencephalic sulci are complex, they may be related to the topography of the folds, expression being generally stronger in those cells near to the convex surface of the folding neural ectoderm as early as E9.5. By E11.5-E12.5, this differential expression has resolved into readily distinguishable

Pax6 expressing and *Pax6* non-expressing cell populations, which are also distinct in terms of their expression of *steel*. Differences in morphology between *Pax6* expressing and non-expressing cells, show that the potential exists for Pax6 to be regulating cell behaviour that is differential across a folding tissue. On the other hand, the suggestion that early differential *Pax6* mRNA expression levels are significant may be difficult to reconcile with the absence of a forebrain shape phenotype in heterozygous *Small eye* mice, predicted to have a 50% reduction in functional PAX6.

6.10 *Pax6* and forebrain segmentation

The vertebrate forebrain has long been recognised as a segmented structure, although only recently has it been shown (Figdor and Stern, 1993) that, like the rhombomeres of the hindbrain (Fraser et al 1990), the units from which the diencephalon is composed are compartments between which there is little cell mixing. Strong *Pax6* expression in developing ventral thalamus has been described before (Walther and Gruss). That the boundaries of this expression, and the expression of many other genes in forebrain, are the boundaries of morphologically recognisable subunits has been used by a number of authors (Figdor and Stern 1993; Puelles and Rubenstein, 1993) as evidence in support of the idea that the observed neuromeres reflect distinct developmental units of a segmented forebrain.

Vertebrate *Pax6* encodes a protein with considerable amino sequence similarity to the products of the *Drosophila* pair-rule gene *paired* , and the *gooseberry* segment polarity genes and has DNA binding motifs almost identical to

those in the product of the *Drosphila Pax6* gene *eyeless*. Embryos homozygous for *paired* mutations have reduced numbers of segments due to deletions of alternate parasegments (Nüsslein Volhard and Wieschaus, 1980; Sander *et a.l*, 1980). The *Drosophila Pax6* gene, *eyeless* also shows a segmented pattern of expression in the developing ventral nervous system (Quiring *et al.*, 1994) and there are distinct blocks of expression in the larval brain. In this case however it is not known what the result of mutations affecting this expression would be, as the existing, viable, mutations only reduce expression in the eye imaginal disc. *Pax6* is thus related to a number of segmentally expressed *Drosophila* genes, some of which are known to be involved in anterior-posterior patterning.

Segment restricted diencephalon expression of *Pax6* mRNA, raises the possibility of altered brain shape resulting from abnormal specification of anterior - posterior position within the forebrain. The observation that levels of *Pax6* mRNA expression vary along the antero-posterior axis early in forebrain development, prior to the time at which forebrain shape defects become apparent, make this explanation more plausible. *Sey/Sey* embryos also lack the normally clear boundary in *Pax6* mRNA expression between forebrain and midbrain, but the scanning electron microscopy analysis suggests that this does not reflect an underlying loss of distinction between forebrain and midbrain and that at least posteriorly in the diencephalon segmentation is not affected. Disrupted antero-posterior patterning remains a possible explanation more anteriorly however. Schmahl *et al.*, (1993) examined later Sey^{Neu} /Sey^{Neu} embryos and observed no gross abnormalities of the thalamus. Thus while there may be defects in anterior diencephalon, it is unlikely for

example that an entire neuromere is absent. The significance of the early segmented nature of *Pax6* expression, if it is not involved in the segmentation process itself, is unclear. One possibility is suggested by the observation that within the fields of *Pax6* expression some cells come to express at a high level. The segmented fields of expression may thus simply be a stage en-route to some regulatory role in a much smaller population of cells. Further analysis of this possibility awaits identification of the strongly expressing cells near the forebrain-midbrain boundary and their phenotype in *Sey/Sey* mutants.

6.11 Loss of *Pax6* mRNA expression from posterior diencephalon.

PAX6-dependant, *Pax6* mRNA expression has been found in the surface ectoderm adjacent to the optic vesicle, and in the nasal placode region (this study, reported in Grindley *et al.,* 1995). In *Sey/Sey* mutants, *Pax6* expression in these regions was downregulated, but expression was retained in the optic vesicle and forebrain, suggesting that the PAX6-dependence of *Pax6* expression was tissue-specific. The absence of *Pax6* mRNA expression from the posterior diencephalon in *Sey/Sey* embryos indicated that this phenomenon also occurs in the neuroepithelium, and the retention of *Pax6* expression in the anterior diencephalon confirms that not all *Pax6* expression is equally PAX6-dependant.

Pax6 expression patterns are complex, and many factors may contribute. In *Sey/Sey* mutants one influence is effectively removed, and the resulting pattern may reveal important features of the other factors involved in *Pax6* regulation. For example, the strong expression in the anterior dorsal midline of the *Sey/Sey*

diencephalon and the lack of a precise caudal boundary to this expression would be consistent with this region being a source of a diffusible signal that positively regulates *Pax6* expression.

The domain in which some cells were observed to come to express at a higher level than their neighbours is also the domain in which *Pax6* expression is most dependant upon PAX6. Does this relationship have any significance? Certainly, establishing distinct cell populations expressing *Pax6* at different levels could rely heavily upon a feedback of PAX6 levels if these populations are close together, as they are in the posterior diencephalon.

6.12 Summary of CNS study

This work is concerned with identifying roles for the *Pax6* gene. Two approaches have been combined in this study. The normal pattern of *Pax6* mRNA expression in the developing brain has been examined by in-situ hybridisation. In addition, an early defect in the shape of the forebrain has been identified and characterised using histological analysis, in-situ hybridisation and scanning electron microscopy.

There are many interesting features of *Pax6* mRNA expression in the brain that may not only reveal potential roles for the *Pax6* gene but may also provide information on the normal organisation of the brain. *Pax6* mRNA expression levels vary along both the antero-posterior and dorso-ventral axes, raising the possibility that PAX6 is involved in patterning along either or both of these axes. The domains defined by *Pax6* expression levels highlight some potentially novel subdivisions of the brain and identify stages at which there are differences in gene expression between previously recognised subdivisions.

Pax6 expression in germinative epithelia is consistent with a role in governing the cellular proliferation, or the transition of neurons from a mitotic to a migratory state. Many sites of *Pax6* expression form part of the olfactory system, and boundaries of *Pax6* expression may define the locations of axon tracts.

No definitive explanation has emerged for the early forebrain shape defects in *Sey/Sey* embryos, but a number of suggestions can be made.

Firstly, removal of nasal placodes gives a similar phenotype. Much of the later *Sey/Sey* phenotype could result from the lack of an olfactory nerve, leading to lack of olfactory bulbs, but the early phenotype is probably not due to absence of cells that normally migrate from the placode. A failure of signalling from the placode is a possibility however, and an effect on the brain from the nasal region could also stem from neural crest migration abnormalities. Abnormal neural crest migration is probably a secondary one, as midbrain neural crest cells were not observed to express *Pax6*.

Secondly, the early *Sey/Sey* phenotype is reminiscent of internal hydrocephaly and the relative timing of formation of a closed brain vesicle and the appearance of the defects would be consistent with a mechanism such as increased cerebrospinal fluid pressure or decreased resistance to this pressure. The latter possibility could also explain these defects only being apparent in the forebrain.

Thirdly, *Pax6* expression within the brain could be important in governing the folding properties of forebrain neural ectoderm. By analogy with the processes involved in neurulation, properties affected in *Sey/Sey* mutants might include rates of proliferation and direction of the division planes, adhesive properties and movement of cells and stereotypical changes in cell size and shape. Some cell behaviour, such as the migration of postmitotic cells, is known to be affected in *Small eye* mutants (Schmahl *et al.,* 1993) and many phenotypes of other *Pax* gene mutants can be explained in terms of proliferation or adhesive properties being affected.

Fourthly, segment restricted *Pax6* expression and the loss of a sharp expression boundary in *Sey/Sey* mutants led to the suggestion that antero-posterior

patterning within the forebrain could involve PAX6. From the SEM analysis performed is unlikely that Pax6 has a role in defining the neuromeres in posterior diencephalon, but may be involved in patterning on a finer scale or more anteriorly.

These explanations are by no means exclusive and overlap to some extent. PAX6 might for example regulate properties that affect the differential resistance to expansion within neural ectoderm, resulting in hydrocephalic embryos. Equally PAX6 could be governing properties of cells near neuromere boundaries, with these properties also being important for the way the neural ectoderm folds. It seems likely that in *Sey/Sey* embryos, there is some effect on the brain from nasal region defects, but additional contributions to the phenotype could come from any of the mechanisms described. Further analysis will help to decide between these, and other, possibilities.

Pax6 expression at the forebrain-midbrain boundary appears to be PAX6 dependant, but other genes that may be regulated by PAX6 in the brain have yet to be identified. This work should enable *Pax6* expression and *Small eye* developmental defects to be related to the expression of other genes, providing candidates for regulation by PAX6. With some suggestions for downstream targets it is possible to reconcile complex features of *Pax6* expression and *Small eye* developmental defects. For example ,the hypothesis that PAX6 regulates adhesion molecules is consistent with *Pax6* expression adjacent to axon tracts, features of the early *Sey/Sey* defects described here and even some features of later neuronal migration defects in *Small eye* mice.

CHAPTER 7.

Suggested improvements and possible future studies

7.1 Some possible improvements to this study.

If these experiments were to be repeated there are many ways in which they could be improved. Some of these could strengthen the studies and perhaps provide considerable additional information. Other suggestions are presented only as examples of alternative practice that might usefully be adopted in a hypothetical future study.

The *Pax6* riboprobe used would detect both the main forms of the *Pax6* message, with and without exon 5a. It would also detect transcripts differing at the 5' end, such as from different promoters. This means that the *Pax6* expression patterns presented here are in some way a composite of expression of different *Pax6* transcripts, and may conceal important temporal or tissue-specific variation in the pattern of different transcripts. It is quite possible that one phenomena discovered in this study, PAX6 dependent *Pax6* mRNA expression, might affect some transcripts more than others, especially if alternative promoters are used. Use of transcript specific probes may thus build on the work presented here and has been initiated (Pen Rashbass, personal communication).

It is still open to debate whether or not the *Sey* allele of *Small eye* is really a null, as the predicted product still has the paired domain intact. Since gain of function effects can not be excluded in this case, the conclusions on normal roles of *Pax6* would be firmer if a null allele were to be used. To produce a 'knock-out' mouse when there are several existing alleles at the same locus, may be an unattractive prospect, but ES cells and mice in which *Pax6* was replaced by a marker such as *lacZ*

or green fluorescent protein would be a useful resource and could make analysis of the type performed here considerably more rapid and precise.

Another possible weakness of the study is the use of untyped *Sey*/+ and +/+ littermates as controls. There are advantages to this: They can be closely matched to the same developmental stage, are subject to far more similar treatment than embryos from different litters, and in principle, use of littermates makes a more economical use of laboratory animals. The clear disadvantage is the difficulty of being sure that there are no significant differences between *Sey*/+ and wild-type animals for the property under consideration. Tissue samples were kept for retrospective PCR analysis of littermates in almost all cases, but this analysis was in practice restricted to the most critical samples. Better practice overall would be to either use only wild-type controls, or to perform a direct comparison of *Sey*/+ and +/+ mice. The undulated mutation provides a pointer to the value of this latter approach. Two undulated alleles were long thought to be recessive, but a recent careful analysis found that they were semidominant, and had subtle and sometimes transient heterozygous phenotypes. For the homozygous *Small eye* defects identified in this study, work specifically aimed at the possibility of mild heterozygous phenotypes may be of some interest. As an example a quantitatively comparison of the size of surface ectoderm *Pax6* expression domains in the eyes of *Sey*/+ and +/+ animals might address both the question of whether autoregulation affected heterozygous *Small eye* mice and the question of whether micropthalmia might be caused by the lens placode region being smaller than normal.

Some of the studies performed have used relatively small numbers of samples. Firmer conclusions could perhaps have been reached either simply by using larger numbers, or by coupling this to a quantification of traits. For example the transmission electron microscopy studies did not have sufficiently complete series of sections to determine whether properties, such as amounts of cell death, that varied with the plane of section and from eye to eye, were any different in mutants and controls.

7.2 Suggested future work.

The results in this study are consistent with an autonomous action of *Pax6* in lens and nasal placode development. *Pax6* certainly has functions, such as a role in its own regulation, that can be separated from the influence of the optic vesicle. Nevertheless it is important to demonstrate the autonomy of *Pax6* action in lens and nasal development. Substantial evidence for this will come from the studies of targets of PAX6. Crystallin gene regulation by PAX6, for example, implies an autonomous *Pax6* action during lens development. Tissue recombination experiments, like those of Fujiwara et al, (1994), could usefully be repeated using a molecular genotyping to allow tissues to be taken before defects were apparent. Another possible approach is the use of chimeras in which *Small eye* mutant cells can be individually identified. This has been used to demonstrate autonomous action of *Pax6* in both lens and retina development (Jane Thornbury, personal communication).

The relationship found in this study, between *Pax6* expression to the capacity to form lens, is difficult to test. *Pax6* expression and ability to transdifferentiate into lens, is one area that might be examined. Activating inducible *Pax6* expression, or

transfecting cells with *Pax6* might be able to confer this ability on tissues that normally lack it. Studies of transdifferentiating cells already show that *Pax6* expression is upregulated early in lentoid body formation (Olaf Sundin and co-workers, personal communication), and Pax6 is also expressed during lens regeneration Del Rio-Tsonis *et al.* (1995). A simple way to further examine the relationship between *Pax6* and capacity to form lens would be to make use of known differences between species. The capacity to form lens in response to optic vesicle grafts is a property of a broad region of head surface ectoderm in most amphibians, but in *Rana esculenta* embryos, lens forming potential is so dramatically restricted that even head ectoderm adjacent to the presumptive lens ectoderm, will not respond to an optic vesicle (reviewed by Saha *et al.*, 1989) Comparison of *Pax6* expression in this and other species could thus be worthwhile.

The study of eye and nasal development here make two suggestions of possible targets for PAX6, *Pax6* itself and the *Msx1* gene. PAX6 dependent *Pax6* mRNA regulation could well be direct autoregulation. A significant *in vivo* target of PAX6 is worth studying in its own right, but also provides a good positive control for molecular approaches to finding other targets. Immunoprecipitation of protein DNA complexes with antibodies against PAX6 should isolate the known PAX6 binding site from the *Pax6* gene. *Msx1* expression is affected only in one specific domain of the transgenic pattern. A deletion analysis of the *Msx*1 promoter could perhaps identify the cis-elements involved in producing this domain of expression. Even if PAX6 is not directed involved in this regulation, intermediate genes, that could be targets of PAX6, might thus be identified.

The work in this thesis on the early forebrain abnormalities of *Sey/Sey* embryos presents some phenomena and suggests mechanisms to explain them, but it has not been possible as yet to decide between these or other mechanisms. In particular it would be desirable to separate the influence of failed nasal development from the remaining phenotype. Chimeras are of limited use for this problem, but would be interesting for studying what contribution *Sey/Sey* mutant cells could make to structures which express *Pax6*, but may fail to form in *Sey/Sey* animals due to other defects. The olfactory bulbs are a good example of this, as defects in them are masked by the consequences of failed olfactory nerve development. For the early brain shape problem it would be useful to create transgenic mice having wild-type PAX6 in the nasal region but not in the brain, and *vice versa*. Hopefully, the former such mice would have nasal cavities and be able to breath, which would also allow the consequences of the later cerebral cortex and cerebellar abnormalities of homozygous embryos to be more effectively analysed.

The relationship between *Pax6* expression and folding of neural ectoderm is difficult to test directly. A search for possible mechanism such as differential cell shape or adhesive properties might produce a correlation with *Pax6* expression and a feature that could be examined in more detail in *Sey/Sey* mutants, or in culture.

An involvement of *Pax6* in antero-posterior patterning of the anterior diencephalon is rather easier to examine. Examination of expression patterns for markers expressed in specific antero-posterior domains in the diencephalon (reviewed by Puelles and Rubenstein, 1993), may reveal whether patterning in the anterior diencephalon is disrupted in line with the abnormal morphology.

BIBLIOGRAPHY

Abney, E. R., Bartlett, P. P. and Raff, M. C. (1981). Astrocytes, ependymal cells, and oligodendrocytes develop on schedule in dissociated cell cultures of embryonic rat brain. *Dev. Biol.* 83, 301-310

Adams B., Dorfler P., Aguzzi A., Kozmik Z., Urbanek P., Maure-Fogy I., and Busslinger M.(1992). *Pax-5* encodes the transcription factor BSAP and is expressed in B lymphocytes, the developing CNS and adult testis. *Genes Dev.* **6,** 1589-1607.

Adler, R. and Hatlee, M. (1989). Plasticity and differentiation of embryonic retinal cells after terminal mitosis. *Science* **243**, 391-393.

Agarwal, V. R. and Sato, S. M. (1993). Retinoic acid affects central nervous system development of *Xenopus* by changing cell fate. *Mech. Dev.* **44**, 167-173.

Allen, N. D., Cran, D. G., Barton, S. C., Hettle, S., Reik, W. and Surani, A. (1988). Transgenes as probes for active chromosomal domains in mouse development. *Nature* **333**, 852-855.

Allison, A. C. (1953). The morphology of the olfactory system in the vertebrates. *Biol. Rev.* **28**, 195-244.

Alvardo-Mallart, R. -.M., Martinez, S. and Lance-Jones, C. C. (1990). Pluripoentiality of 2-day-old avian germinative neuroepithelium. *Dev Biol.* **139**, 75-88 .

Ang, S. L. and Rossant, J. (1994). HNF-3β is essential for node and notochord formation in mouse development. *Cell* **78**, 561-574.

Asano, M. and Gruss, P. (1992). *Pax-5* is expressed at the midbrain-hindbrain boundary during mouse development. *Mech. Dev.* **39**,29-39.

Auerbach R. (1954). Analysis of the developmental effects of a letal mutation in the house mouse. *J. Exp. Zool* **127**, 305-329.

Baldwin, C. T. , Hoth, C. F., Amos, J. A., da Silva, E. O. and Milunsky, A. (1992). An exonic mutation in the *HuP2* paired domain gene causes Waardenburg's syndrome. *Nature* **355**, 637-638.

Balling, R., Deutsch, U. and Gruss, P. (1988). *Undulated*, a mutaion affecting the development of the mouse skeleton, has a point mutation in the paired box of *Pax1*. *Cell* **55**, 531-535.

Barabanov, V. M. and Fedtsova, N. G. (1982). The distribution of lens differentiation capacity in the head ectoderm of chick embryos. *Differentiation* **21,** 183-190.

Barbe, M. F. and Levitt, P. (1991). The early commitment of fetal neurons to the limbic cortex. *J. Neurosci.* **11**, 519-533.

Barberis A., Superti-Furga, G., Vitelli, L., Kelmer, I. and Busslinger, M. (1989). Developmental and tissue-specific regulation of a novel transcription factor of the sea urchin. *Genes Dev.* **3**, 663-675.

Barberis, A., Widenhorn, K., Vitelli, L. and Busslinger, M.(1990). A novel B-cell lineage specific transcription factor present at early but not late stages of differentiation. *Genes Dev.* **4**, 849-859.

Barishak, R. (1992). Embryology of the eye and its adenexa. *Dev. Opthalmol.* **24.**

Barr, F. G., Galili, N.Holick, J., Biegel, J. A., Rovera, G. and Emmaunuel, B. S. (1993). Rearrangement of the PAX3 paired box gene in the pediatric solid tumor alveolar rhabdomyosarcoma. *Nature Genet.* **3**, 113-117.

Basler, K., Edlund, T., Jessell, T. M. and Yamada, T. (1993). Control of cell pattern in the neural tube: regulation of cell differentiation by dorsalin-1, a novel TGFβ family member. *Cell* **73**, 687-702.

Baumgartner, S. and Noll, M. (1990). Network of interactions amoung pair-rule genes regulating *paired* expression during primodial segmentation of *Drosophila. Mech Dev.* **33**, 1-18.

Beddington, R. S. P. and Lawson, K. A. (1990).In *Postimplantation mammalian embryos: a practical approach* (eds. Copp, A. J. and Cockroft, D. L.) pp 267-292. Oxford University Press.

Beddington, R. S. P. (1994). Induction of a second neural axis by the mouse node. *Development* **120**, 613-620.

Beebe, D. C., Compart, P. J., Johnson, M. C., Feagans, D. E. and Feinberg, R. N. (1982). The mechanism of cell elongation during lens fiber cell differentation. *Dev. Biol.* **92**, 54-59.

Begleiter, M. L. and Harris, D. J. (1992). Waardenburg syndrome and meningocele. *Am J. Hum. Genet.* **44**,541.

Bell, E. T. (1906). Experimental studies on the development of the eye and nasal cavities in frog embryos. *Anat Anz.* **29**, 185-194.

Bell, E. T. (1907). Some experiments on the development and regeneration of the eye and nasal organ in frog embryos. *Arch. Entw. Mech. d. Org.* **23**, 645-678.

Bellen, H. J., Wilson, C. and Gehring, W. J. (1990). Dissecting the complexity of the nervous system by enhancer detection. *BioEssays* **12**, 199-204.

Bergquist, H. and Källén, B. (1954). Notes on the early histogenesis and morphogenesis of the central nervous sytem in vertebrates. *J. Comp. Neurol.* **100**, 627-659.

Birgbauer, E. and Fraser, S. E. (1994). Violation of cell lineage restriction compartments in the chick hindbrain. *Development* **120**, 1347-1356.

Bober, E., Franz, T., Arnold, H. H., Gruss, P. and Tremblay, P.(1994). *Pax-3* is required for the development of limb muscles: a possible role for the migration of dermomyotomal muscle progenitor cells. *Development* **120**, 603-12.

Bopp, D., Burri, M., Baumgartner, S., Frigerio, G. and Noll, M. (1986). Conservation of a large protein domain in the segmentation gene *paired* and in functionally related genes of *Drosophila. Cell* **47**, 1033-1040

Bopp, D., Jamet, E., Baumgartner, S., Burri, M. and Noll, M. (1989). Isolation of two tissue-specific *Drosophila* paired box genes *Pox meso* and *Pox neuro. EMBO J.* **8**, 3447-3457

Bovolenta P. and Dodd, J. (1991). Perturbation of neuronal differentiation and axon guidance in the spinal chord of mouse embryos lacking a floor plate: analysis of *Danforth's short-tail* mutation. *Development* **113**, 625-639.

Bradley, R. S., Cowin, P. and Brown, A. M. C. (1993). Expression of *Wnt-1* in PC12 cells results in modulation of *Plakoglobin* and *E-Cadherin* and increased cellular adhesion. *J. Cell Biol.* **123**, 1857-1865.

Brady R. C. and Hilfer, S. R. (1982). Optic cup formation: a calcium regulated process.
Proc. Natl. Acad. Sci. USA **79**, 5587-5591.

Brand-Saberi, B., Ebensperger, C., Wilting, J., Balling, R. and Christ, B. (1993). The ventralizing effect of the notochord on somite differentiation. *Anat. Embryol* **188**, 239-245.

Buck, L. and Axel, R. (1991). A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell* **65**, 175-187.

Burr, H.S.(1916). The effects of removal of the nasal pits in *Amblystoma* embryos. *J. exp. Zool.* **20**, 27-57.

Burri, M., Tromvoukis, Y., Bopp, D., Frigerio, G. and Noll, M. (1989). Conservation of the paird domain in metazoans and its structure in three isolated human genes. *EMBO J.* **8**, 1183-1190.

Byrd, C. A. and Burd, G. D. (1993).Morphological and quantitative evaluation of olfactory bulb development in *Xenopus* after olfactory placode transplantation. *J. Comp. Neurol.* **331**, 551-563.

Cabelli, R. J., Hohn, A. and Shatz, C. J. (1995). Inhibition of ocular dominance column formation by infusion of NT-4/5 or BDNF. *Science* **267**, 1662-1666.

Call, K. M., Glaser, T., Ito, C. Y., Buckler, A. J., Pelletier, J., Haber, D. A., Rose, E. A., Kral, A., Yeger, H., Lewis, W. H., Jones, C. and Houseman, D. E. (1990). Isolation and characterisation of a zinc-finger gene at the human chromosome 11 Wilms' tumor locus. *Cell* **60**, 509-520.

Chalepakis, G., Fritsch, R., Fickenscher, H., Deutsch, U., Goulding, M. and Gruss P. (1991). The molecular bais of the *undulated /Pax-1* mutation. *Cell* **66**, 873-884.

Chen, Z. -F. and Behringer, R. B. (1995). Twist is required in head mesenchyme for cranial neural tube morphogenesis. *Genes Dev.* **9**, 686-699

Clairambault, P. (1968). Morphegenèse du télencéphale: morphegenèse expérimentale du télencéphale des Ranidés. *Arch. Biol. (Liège)* **79**, 537-578.

Clayton, R. M. and Campbell, J. C. (1968). *Small eye*, a mutant in the house mouse apparently affecting the synthesis of extracellular membranes. *J. Physiol*, **198**, 74P-75P.

Clarke, J. D. W., Holder, N., Soffe, S. R. and Storm-Mathisen, J. (1991). Neuroanatomical and functional analysis of neyural tube formation in notochordless *Xenopus* embryos; laterality of the ventral spinal chord is lost. *Development* **112**, 499-516.

Colamarino, S. A. and Tessier-Lavigne, M. (1995). The axonal chemoattractant netrin-1 is also a chemorepellant for trochlear motor axons. *Cell* **81**, 61-629.

Colello, R. J. and Guillery, R. W. (1990). The early development of retinal ganglion cells with uncrossed axons in the mouse: retinal position and axonal course. *Development* **108**, 515-523.

Colello, R. J. and Guillery, R. W. (1992). Observations on the early development of the optic nerve and tract of the mouse. J. Comp. Neurol. 317, 357-378.

Coulombre, A. J. (1965). The Eye, *in* "Organogenesis" (R. L. DeHaan and H. Ursprung, Eds.), pp 219-251. Holt, Rinehart and Winston, New York.

Coulombre, A. J. and Coulombre, J. L. (1964). Lens development: I. Role of the lens in eye growth. *J. Exp. Zool.* **156**, 39-48.

Crossley, P. H. and Martin, G. R. (1995). The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning of the developing embryo. *Development* **121**, 439-451.

Cushieri, A. and Bannister, L. H. (1975). The development of the olfactory mucosa in the mouse: light microscopy. *J. Anat.* **119**, 277-286.

Dambly-Chaudiere, C., Jamet, E., Burri, M., Bopp, D., Basler, K., Hafen, E., Dumont, N., Speilmann, P., Ghysen, A. and Noll, M. (1992). The paired box gene *pox neuro*: A determinant of poly-innervated sense organs. *Cell* **69**, 159-172.

Davis, A. and Cowell, J. K. (1994). Mutations in the **PAX6** gene in patients with hereditary aniridia. *Hum. Mol. Genet.* **2**, 2093-2097

Davis, R. J., D'Cruz, C. M., Lovell, M. A., Biegel, J. A. and Barr, F. G. (1994). Fusion of PAX7 to FKHR by the variant t(1:13)(p36;q14) translocation in alveolar rhabdosarcoma. *Cancer Res.* **54**, 2869-2872.

Del Rio-Tsonis, K., Washabaugh, C. H. and Tsonis, P. A. (1995). Expression of *pax-6* during urodele eye developmeny and lens regeneration. *Proc. Natl. Acad. Sci. USA* **92**, 5092-5096.

DeMeyer, W. (1975). Median facial malformations and their implications for brain malformations. *Birth Defects: Original article series* **XI**, 155-181.

Desmond, M. E. and Jacobson, A. G. (1977). Embryonic brain enlargement requires cerebrospinal fluid pressure. *Dev. Biol.* **57**, 188-198.

Deutsch, U., Dressler, G. R. and Gruss, P. (1988). *Pax1*, a member of a paired box homologous gene family, is expressed in segmented structures during development. *Cell* **53**, 617-625.

Diakoku-Ishido, H., Okamura, Y., Yanaihara, N. and Daikoku, S. (1990). Development of the hypothalamic luteinizing hormone-releasing hormome-containing neuron system in the rat: *in vivo* and in transplantation studies. *Dev. Biol.* **140**, 374-387

Dietich, S., Schubert, F. R. and Gruss, P. (1993). Altered Pax gene expression in murine notochord mutants: the notochord is required to initiate and maintain ventral identity in the somite. *Mech Dev.* **44**, 189-207

Dietrich, S. and Gruss, P. (1995). *Undulated* phenotypes suggest a role of *Pax-1* for the development of vertebral and extravertebral structures. *Dev. Biol.* **167**, 529-48.

Dong, L.-J., and Chung, A. E. (1991). The expression of the genes for entactin, laminin A, laminin B1 and laminin B2 in murine lens morphogenesis and eye development. *Differentiation* **48**, 157-172.

Dorshkind, K. (1994). Transcriptional control points during lymphopoesis. *Cell* **79**, 751-753.

Doucette, R. (1989). Development of the nerve fiber layer in the olfactory bulb of the mouse. *J Comp.Neurol.* **285**, 514-527.

Dressler, G. R., Deutsch, U., Chowdhury, K., Nornes, H. O. and Gruss, P. (1990). *Pax2*, a new murine paired-box containing gene and its expression in the developing excretory system. *Development* **109**, 787-795.

Dressler, G. R. and Douglass, E. C. (1992). Pax-2 is a DNA-binding protein expressed in embryonic kidney and Wilms tumor. *Proc. Natl. Acad. Sci. USA* **89,** 1179-1183.

Dressler, G. R., Wilkinson, J. E., Rothenpieler, U. W., Patterson, L. T., Williams-Simons, L. and Westphal, H. (1993). Deregulation of *Pax-2* expression in transgenic mice generates severe kidney abnormalities. *Nature* **362**, 65-7

Durston, A. J., Timmermans, J. P. M., Hage, W. J., Hendricks, H. F. J., de Vries, N. J., Heideveld, M. and Nieuwkoop, P. D. (1989). Retinoic acid causes an anteroposterior transformation in the developing nervous system. *Nature* **340**, 140-144.

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A. and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicates in the regulation of CNS polarity. *Cell* **75**, 1417-1430.

Epstein D.J., Vekemans M., and Gros P. (1991). *Splotch* (Sp^{2H}), a mutation affecting development of the mouse neural tube, shows a deletion within the paired homeodomain of *Pax-3. Cell* **67**, 767-774.

Epstein, D. J., Vogan, K. J., Trasler, D. G. and Gros-P. (1993). A mutation within intron 3 of the *Pax-3* gene produces aberrantly spliced mRNA transcripts in the *splotch* (*Sp*) mouse mutant. *Proc.Natl. Acad. Sci. USA.* **90**, 532-6.

Epstein, J., Cai, J., Glaser, T., Jepeal, L. and Maas, R. (1994a). Identification of a Pax paired domain recognition sequence and evidence for DNA depedent conformational change. *J. Biol. Chem.* **269**, 8355-8361.

Epstein, J. A., Glaser, T., Cai, J., Jepeal, L., Walton, D. S. and Maas, R. L. (1994b). Two independent and interactive DNA-binding subdomians of Pax6 paired domain are regulated by alternative splicing. *Genes Dev.* **8**, 2022-2034.

Fan, C. -M. and Tessier-Lavigne, M. (1994). Patterning of mammalian somites by surface ectoderm and notochord: evidence for scleratome induction by a hedgehog homolog. *Cell* **79**, 1175-1186.

Farrer, L. A., Arnos, K. S., Asher, J. H. Jr., Baldwin, C., T., Diehl, S. R., Friedman, T. B., Greenberg, J., Grundfast, K. M., Hoth, C., Lawani, A. K., Landa, B.,, Leverton, K., Milunsky, A., Morell, R., Nance, W. E., Newton, V., Ramesar, R., Rao, V. S., Reynolds, J. E., San Agustin, T. B., Wilcox, E. R., Winship, I. and Read, A. P. (1994). Locus heterogeneity for Waardenburg syndrome is predictive of clincal subtypes. *Am J. Hum. Genet.* **55**, 728-737.

Ferri, R. T. and Levitt, P. (1993). Cerebral cortical progenitors are fated to produce region-specific neuronal populations. *Cerebral Cortex* **3**, 187-198.

Figdor, M.C. and Stern, C.D. (1993). Segmental organisation of embryonic diencephalon. *Nature* **363**, 630-634.

Findlater, G. S., McDonald, R. D. and Kaufman, M. H. (1993). Eyelid development, fusion and subsequent reopening in the mouse. *J. Anat.* **183**, 121-129.

Fishell, G., Mason, C. A. and Hatten, M. E. (1993). Dispersion of neural progenitors within the germinal zones of the forebrain. *Nature* **362**, 636-638.

Fraser, S. Keynes, R. and Lumsden, A. (1990). Segmentation in the chick embryo hindbrain is defined by cell lineage restrictions. *Nature* **344**, 431-435.

Franco, B., Gioli, S., Pragliola, A., Incerti, B., Bardoni, B., Tonlorenzi, R., Carrozzo, R., Maestrini, E., Pieretti, M., Taillon-Miller, P., Brown, C. J., Wilard, H. F., Lawrence, C., Persico, M. G., Camerino, G. and Ballabio, A. (1991). A gene deleted in Kallman's syndrome shares homolgy with neural cell adhesion and axonal path-finding molecules. *Nature* **353**, 529-536.

Franz, T. (1989). Persistent truncus arteriosus in the *Splotch* mutant mouse. *Anat. Embryol.* **180**, 457-464.

Franz, T. (1990). Defective ensheathmant of motoric nerves in the *Splotch* mutant mouse. *Acta. Anat.* **138**, 246-253.

Franz, T. (1992). Neural tube defects without neural crest defects in *Splotch* mice. *Teratology* **46**, 599-604.

Franz, T. (1993). The *Splotch* (Sp^{1H}) and *Splotch-delayed* (Sp^{d}) alleles: differential phenotypic effects on neural crest and limb musculature. *Anat-Embryol-Berl.* **187**, 371-377

Franz, T. (1994). *Extra-toes* (*Xt*) homozygous mutant mice demonstrate a role for the *Gli-3* gene in the development of the forebrain. *Acta. Anat.* **150**, 38-44.

Franz, T., Kothary, R., Surani, M. A., Halata, Z. and Grim, M. (1993). The *Splotch* mutation interferes with muscle development in the limbs. *Anat-Embryol-Berl.* **187**, 153-160.

Fredericks, W. J., Galili, N., Mukhopadhyay, S., Rovera, G., Bennicelli, J., Barr, F. G. and Rauscher, F. J. 3rd (1995). The PAX3-FKHR fusion protein created by the t(2;13) translocation in alveolar rhabdomyosarcomas is a more potent transcriptional activator than PAX3. *Mol.Cell Biol.* **15**, 1522-1535.

Friesel, R. and Brown, S. A. N. (1992). Spatially restricted expression of fibroblast growth factor receptor-2 during *Xenopus* development. *Development* **116**, 1051-1058.

Fujiwara, M., Uchida, T., Osumi-Yamashita, N. and Eto, K. (1994). Uchida rat (*rSey*): a new mutant rat with craniofacial abnormalities resembling those of the mouse *Sey* mutant. *Differentiation* **57**, 31-38.

Gaze, R. M., Feldman, J. D., Cooke, J. and Chung, S.-H. (1979). The orientaion of the visuotectal map in *Xenopus*: Developmental aspects. *J Embryol. Exp. Morphol.* **53**, 39-66.

Gehring, W. J., Müller, M., Affolter, M., Percival-Smith, A., Billeter, M., Qian, Y. Q., Otting, G. and Wüthrich, K. (1990). The structure of the homeodomain and its functional implications. *Trends Genet.* **6**, 323-329.

Gessler, M., Poustka, A., Cavenee, W., Neve, R. L., Orkin, S. H. and Bruns, G. A. P. (1990). Homozygous deletion in Wilms tumours of a zinc-finger gene identified by chromosome jumping. *Nature* **343**, 774-778.

Ghysen, A. and Dambly-Chaudiere, C. (1993). The specification of sensory neuron identity in *Drosophila*. *Bioessays* **15**, 293-298.

Gaze, R. M., Feldman, J. D., Cooke, J. and Chung, S. -H. (1979a). The orientaion of the visuotectal map in *Xenopus*: developmental aspects. *J Embryol. Exp. Morphol.* **53**, 39-66.

Gilbert, C. D. and Wiesel, T. N. (1979). Morphology and intracortical projections of functionally characterised neurones in the cat visual cortex. *Nature* **280**, 120-125.

Glaser, T., Walton, D. S., and Maas, R. L. (1992). Genomic structure, evolutionary conservation and aniridia mutations in the human *PAX6* gene. *Nature Genet.* **2,** 915-920.

Glaser, T.,Jepeal, L., Edwards, J. G., Young, S. R., Favor, J. and Maas, R. L. (1994). PAX6 gene dosage effect in a family with congenital cataracts, aniridia, anophthalmia and central nervous system defects. *Nature Genet.* **7,** 463-71.

Glaser, T., Walton, D. S., Cai, J., Epstein, J. A., Jepeal, L. and Maas, R. L. (1995). *PAX6* gene mutations in aniridia. *In*, "Molecular Genetics of Ocular Disease" (J. L. Wiggs, Ed.), pp 51-82. Wiley-Liss, New York.

Gong, Q. and Shipley, M. T. (1995). Evidence that pioneer olfactory axons regulate telencephalon cell cycle kinetics to induce formation of the olfactory bulb. *Neuron* **14**, 91-101.

Goodman, C. S. and Shatz, C. J. (1993). Developmental mechanisms that generate precise patterns of neuronal connectivity. *Cell* **72/***Neuron* **10 (Suppl.)**, 77-98.

Goulding, M. D., Chalepakis, G., Deutsch, U., Erselius, J. R. and Gruss, P. (1991). *Pax-3,* a novel murine DNA-binding protein expressed during early neurogenesis. *EMBO J.* **10**, 1135-1147.

Goulding M.D., Lumsden A. and Gruss P. (1993a). Signals from the notochord and floor plate regulate the region-specific expression of two *Pax* genes in the developing spinal chord. *Development* **117,** 1001-1016.

Goulding, M., Sterrer, S., Fleming, J., Balling, R., Nadeau, J., Moore, K. J; Brown, S. D., Steel, K. P. and Gruss, P. (1993b). Analysis of the *Pax-3* gene in the mouse mutant *Splotch. Genomics* **17**, 355-63.

Graham, A., Heyman, I. and Lumsden, A.(1993). Even-numbered rhombomeres control the apoptotic elimination of neural crest cells from odd-numbered rhombomeres in the chick hindbrain. *Development* **119**, 233-245.

Grainger, R. M. (1992). Embryonic lens induction: shedding light on vertebrate tissue determination. *Trends. Genet.* **8**, 349-355.

Grainger, R. M., Henry, J. J. and Henderson, R. H. (1988). Reinvestigation of the role of the optic vesicle in embryonic lens induction. *Development* **102**, 517-526.

Grainger, R. M., Henry, J. J., Saha, M. S. and Servetnick, M. (1992b). Recent progress on the mechanisms of embryonic lens formation. *Eye* **6**, 117-122.

Gray's Anatomy (1973). 35th edition, (R. Warwick and P.L. Williams, Eds). Longman Group Ltd, Edinburgh.

Graziadei and Monti-Graziadei. (1985). Continous nerve cell renewal in the olfactory system. *In* "Handbook of Sensory Physiology. Vol IX", (Rubell ,Ed.), pp 55-83. Springer, Berlin.

Graziadei, P. P. and Monti-Graziadei, A. G. (1992). The influence of the olfactory placode on the development of the telencephalon in *Xenopus laevis*. *Neurosci.* **46**, 617-629.

Grindley, J. C., Davidson, D. R. and Hill, R. E. (1995). The role of *Pax-6* in eye and nasal development. *Development* **121**, 1433-1442.

Grove, E. A. (1992). Patterning the developing cerebral cortex. *Curr. Biol.* **2**,142-144.

Grove, E. A., Williams, B. P., Li, D. Q., Hajihosseini, M., Friedrich, A. and Price, J. (1993). Multiple restricted lineages in the embryonic rat cerebral cortex. *Development* **117**, 553-61.

Grüneberg, H. (1954). Genetical studies on the skeleton of the mouse XII. The development of *undulated*. *J. Genet.* **52**, 441-455

Gundersen, R. W. (1987). Response of sensory neurites and growth cones to patterned substrata of laminin and fibrotectin *in vitro*. *Dev. Biol.* **121**, 423-431.

Guthrie S. and Lumsden A. (1991). Formation and regeneration of rhombomere boudaries in the devloping chick hindbrain. *Development* **112**,221-230.

Guthrie-S. (1992). Lineage in the cerebral cortex: when is a clone not a clone? *Trends-Neurosci.* **15**, 273-5.

Guthrie, S., Prince, V. and Lumsden, A. (1993). Selective dispersal of avian rhombomere cells in orthotopic and heterotopic grafts. *Development* **118**, 527-538.

Gutjahr, T., Patel, N. H., Li, X., Goodman, C. S. and Noll, M. (1993). Analysis of the *gooseberry* locus in *Drosophila* embryos: *gooseberry* determines cuticular pattern and activates *gooseberry* neuro. *Development* **118**, 21-31.

Gurdjian, E. S. (1925). Olfactory connections in the albino rat with special reference to the stria medullaris and the anterior commisure. *J. Comp. Neurol.* **38**, 127-163.

Halder, G., Callaerts, P. and Gehring, W. J. (1995). Induction of ectopic eyes by targeted expression of the *eyeless* gene in *Drosophila*. *Science* **267**, 1788-1792.

Hallonet, M. E., Teillet, M. A. and Le Dourin, N. M. (1990). A new approach to the development of the cerebellum provided by the quaiil-chick marker system. *Development* **108**, 19-31.

Hanson, I. M, Seawright, A., Hardman, K., Hodgson, S., Zaletayev, D., Fekete, G. and van Heyningen, V. (1993). *PAX6* mutations in aniridia. *Hum. Molec. Genet.* **2**, 915-920.

Hanson, I. M., Fletcher, J. M., Jordan, T., Brown, A., Taylor, D., Adams, R. J., Punnett, H. and van Heyningen, V. (1994a). Mutations at the *PAX6* locus are found in heterogeneous anterior segment malformations including Peters' anomaly. *Nature Genet.* **6**, 168-173.

Hanson, I., Jordan, T. and van Heyningen, V. (1994b). 'Aniridia'. *In* 'Molecular genetics of inherited eye disorders' (A. F. Wright and B. Jay, Eds), pp445-467. Harwood Academic Publishers, Chur, Switzerland.

Hanson, I. and van Heyningen, V. (1995). Pax6: more than meets the eye. *Trends Genet.* **11**, 268-272.

Hatta, K., Kimmel, C. B, Ho, R. K. and Walker, C. (1991). The cyclops mutation blocks specification of the floor plate of the zebrafish central nervous system. *Nature* **350**, 339-341.

Hatta, K., Puschel, A. W. and Kimmel, C. B. (1994). Midline signaling in the primordium of the zebrafish anterior cenral nervous system. *Proc. Natl. Acad. Sci. USA.* **91**, 2061-2065.

Harland, R. M. (1994). Neural induction in *Xenopus. Curr. Opin. Gen. Dev.* **4**, 543-549.

Harrison, J. L. (1958). Some hypoplastic modifications on the telencephalon following unilateral excision of the nasal placode in *Rana pipiens* embryos. *Diss. Abstr. State Univ. Iowa.* **19**, 604.

Heinzmann U., Favor J., Plendl J., and Grevers G. (1991). Enticklungsstorung des olfaktorischen Organs. Ein Beitrag zur kausalen Genese beieiner Mausmutante. *Verh. Anat. Ges.* **85** (*Anat. Anz. Suppl.* **170**), 511-512.

Henry, J. J. and Grainger, R. M. (1987). Inductive interactions in the spatial and temporal restriction of lens-forming potential in embryonic ectoderm of *Xenopus laevis. Dev. Biol.* **24**, 200-214.

Henry, J. J. and Grainger, R. M. (1990). Early tissue interactions leading to embryonic lens formation in *Xenopus laevis. Dev. Biol* **141**, 149-163.

Heyman, I., Kent, A. and Lumsden-A. (1993). Cellular morphology and extracellular space at rhombomere boundaries in the chick embryo hindbrain. *Dev. Dyn.* **198**, 241-53

Hill, R. E., Jones, P. F. Rees, A. R., Sime, C. M., Justice, M. J., Copeland, N. G., Jenkins, N. A., Graham, E. and Davidson, D. R. (1989). A new family of mouse homeo box-containing genes: molecular structure, chromosomal location and developmental expression of *Hox-7.1*. *Genes and Development* **3**, 26-37.

Hill, R. E., Favor, J., Hogan, B. L. M., Ton, C. C. T., Sauders, G. F., Hanson, I. M., Prosser, J., Jordan, T., Hastie, N. D. and van Heyningen V. (1991). Mouse *Small eye* results from mutaions in a *paired*-like homeobox-containing gene. *Nature* **354**, 522-525.

Hinds, J. W. (1972a). Early differentiation in the mouse olfactory bulb. I. Light microscopy. *J Comp. Neurol.* **146**, 233-252.

Hinds, J. W. (1972b). Early differentiation in the mouse olfactory bulb. II. Electron microscopy. *J Comp. Neurol.* **146**, 253-276.

Hirano, S., Fuse, S. and Sohal, G. S. (1991). The effect of the floor plate on pattern and polarity in the developing central nervous system. *Science* **251**, 310-313.

Hittner, H. M., Riccardi, V. M., Ferrell, R. E., Borda, R. R. and Justice, J. Jr. (1980). Variable expresivity in autosomal dominant aniridia by clinical, electrophysiologic and angiographic criteria. *Am J. opthalmol* **89**, 531-539.

Hodgson, S. V. and Saunders, K. E. (1980). A probable case of the homozygous condition of the aniridia gene. *J Med. Genet.* **6**, 478-480

Hogan, B. L. M., Horsburgh, G., Cohen, J., Hetherington ,C. M., Fisher, G. and Lyon, M. F. (1986). *Small eyes* (*Sey*): a homozygous lethal mutation on chromosome 2 which affects the differentiation of both lens and nasal placodes in the mouse. *J. Embryol. exp. Morph.* **97**, 95-110.

Hogan, B. L. M., Hirst, E. M A., Horsburgh, G. and Hetherington, C. M. (1988). *Small eye* (*Sey*): a mouse modeel for the gentic analysis of craniofacial abnormalities. *Development* **103 Suppl.**, 115-119.

Holt, C. (1980). Cell movements in *Xenopus* eye development. *Nature* **287**, 850-852.

Holt, C. E., Bertsch, T. W., Ellis, H. M. and Harris, W. A. (1988). Cellular determination in the *Xenopus* retina is independent of lineage and birth date. *Neuron* **1**, 15-26.

Hoth, C. F., Milunsky, A., Lipsky, N., Sheffer, R., Clarren, S. K. and Baldwin, C. T. (1993). Mutations in the paired domain of the human *PAX3* gene cause Klien-Waardenburg Syndrome (WS-III) as well as Waardenburg syndrome type I (WS-I). Am J. *Hum. Genet.* **52**, 455-462.

Hynes, R. O. and Lander, A. D. (1992). Contact and adhesive specificities in the associations, migrations and targgeting of cells and axons. *Cell* **68**, 303-322.

Hynes, M.; Poulsen, K.; Tessier-Lavigne, M. and Rosenthal, A. (1995). Control of neuronal diversity by the floor plate: contact-mediated induction of midbrain dopaminergic neurons. *Cell* **80**, 95-101.

Jacobson, A. G. (1963a). The determination and positioning of the nose, lens and ear. I. Interactions within the ectoderm, and betwen the ectoderm and underlying tissues. *J. Exp. Zool.* **154**, 273-283.

Jacobson, A. G. (1963b). The determination and positioning of the nose, lens and ear. II. The role of the endoderm. *J. Exp. Zool.* **154**, 285-291.

Jacobson, A. G. (1963c). The determination and positioning of the nose, lens and ear. III. Effects of reversing the antero-posterior axis of the epidermis, neural plate and neural fold. *J. Exp. Zool.* **154**, 293-304.

Jacobson, A. G. (1966). Inductive processes in embryonic development. *Science* **152**, 25-34.

Jacobson, A. G. (1981). Morphogenesis of the neural plate and tube. *In* "Morphogenesis and Pattern Formation", (T. G. Connelly, L. L. Brinkley and B. M. Carlson, Eds.) pp 233-263. Raven Press, New York..

Jacobson, A. G. and Sater, A. K. (1988). Features of embryonic induction. *Development* **104**, 341-359.

Jacobson, A. G. and Tam, P. P. L. (1982). Cephalic neurulation in the mouse embryo analysed by SEM and morphometry. *Anat. Rec.* **203**, 375-396.

Jacobson, M. and Klein, S. L. (1985). Analysis of clonal restriction of cell mingling in *Xenopus*. *Phil. Trans. Roy. Soc. (Lond.) B* **312**, 57-65.

Jacobson, M. and Hiroshi, G. (1978). Origin of the retina from both sides of the embryonic brain: A contribution to the problem of crossing at the optic chiasma. *Science* 202, 637-639.

Jennes, L. (1989). Prenatal development of gonadatrophin -releasing hormone containing systems in the rat brain. *Brain Res.* **482**, 97-108.

Jessell, T. M. and Melton, D. A. (1992). Diffusible factors in vertebrate embryonic induction. *Cell* **68**, 257-270.

Johnston, M. C., Noden, D. M., Hazelton, R. D., Coulombre, J. L. and Coulombre, A. J. (1979). Origins of avian ocular and periocular tissues. *Exp. Eye. Res.* **29**, 27-43.

Johnson, R. L., Laufer, E., Riddle, R. D. and Tabin, C. (1994). Ectoptic expression of sonic hedgehog alters dorso-ventral patterning of somites. *Cell* **79**, 1165-1173.

Jordan, T., Hanson, I., Zaletayev, D., Hodgson, S., Prosser, J., Seawright, A., Hastie, N., and van Heyningen, V. (1992). The human *PAX6* gene is mutated in two patients with aniridia. *Nature Genet.* **1**, 328-332.

Jostes, B., Walther, C. and Gruss, P. (1991). The murine paired box gene, *Pax7*, is expressed specifically during the development of the nervous and muscular system. *Mech. Dev.* **33**, 27-38.

Juriloff, D.M., Sulik, K.K., Roderick, T.H. and Hogan , B.K. (1985). Genetic and developmental studies of a new mouse mutation that produces otocephaly. *J. Craniofac. Genet.* **5**, 121-145.

Kahle, W. (1986). *Color Atlas and Textbook of Human Anatomy. Volume 3: Nervous System and Sensory Organs.* [Translated by Dayan, H.L, and Dayan, A. D.]. Geog Theime Verlag, Stuttgard, New York.

Källén B. (1952). Notes on the proliferation processes in the neuromeres in vertebrate embryos. *Acta Soc. Med. Upsalien* **57**, 111-118

Källén B. (1962). Mitotic patterning in the central nervous system of chick embryos; studied by a chochicine method. *Z. Anat. Entwicklungsgeschichte* **123**, 309-319

Källén B. (1965). Early morhpogenesis and pattern formation in the central nervous system. *In* "Organogenesis" (R. L. DeHaan and H. Ursprung, Eds.), pp107-128. Holt, Rinehart and Winston, New York.

Källén B. and Lindskog, B. (1953). Formation and dissapearance of neuromery in *Mus musculus*. *Acta Anat.* **18**, 273-282.

Karkinen-Jääskeläinen, M. (1978). Permissive and directive interactions in lens induction. *J. Embryol. Exp. Morphol.* **44**, 167-179.

Kato, K. (1992). Finding new genes in the nervous system by cDNA analysis. *Trends Neurosci.* **15**, 319-323.

Kaufman, M. (1979). Cephalic neurulation and optic vesicle formation in the early mouse embryo. *Am J. Anat.* **155**, 425-444.

Kaufman, M.H. (1983). Occlusion of the neural lumen in early mouse embryos analysed by light and electron microscopy. *J. Embryol. exp. Morph.* **78**, 211-228.

Kaufman, M. (1992) *The Atlas of Mouse Development.* Academic Press Ltd, London.

Keller, R., Shih, J. and Sater, A. (1992). The cellular basis of the convergence and extension of the *Xenopus* neural plate. *Dev. Dynam.* **193**, 199-217.

Keller, S. A., Jones, J. M., Boyle, A., Barrow, L. L., Killen, P. D., Green, D. G., Kapousta, N. V., Hichcock, P. F., Swank, R. T. and Meisler, M. H. (1994). Kidney and retinal defects (*Krd*), a transgene induced mutation with a deletion of mouse chromosome 19 that includes the *Pax2* locus. *Genomics* **23**, 309-320.

Kennedy, T. E., Serafini, T., de la Tore, J. R. and Tessier-Lavigne, M. (1994). Netrins are diffusible chemotropic factors for commisular axons in the embryonic spinal chord. *Cell* **788**, 425-435.

Kilchherr, F., Baumgartner, S., Bopp, D., Frei., E. and Noll, M. (1986). Isolation of the paired gene of *Drosophila* and its spatial expression during embryogenesis. *Nature* **321**, 493-499.

Kioussi, C. and Gruss, P. (1994). Differential induction of Pax genes by NGF and BDNF in cerebellar primary cultures. *J. Cell Biol.* **125**, 417-425.

Kollros, J. J. (1982). Peripheral control of midbrain mitotic activity in the frog. *J. Comp. Neurol.* **205**, 171-178.

Koseki, H., Wallin, J., Wilting, J., Mizutani, Y., Kispert, A., Ebensperger, C., Herrmann, B. G., Christ, C., and Balling, R. (1993). A role for Pax-1 as a mediator of notochordal signals during the dorsoventral specification of vertebrae. *Development* **119**, 649-660.

Kozmik, Z., Wang, S., Dorfler, P. Adams, B. and Busslinger, M. (1992). The promoter of the CD19 gene is a target for the B cell -specific transcription factor BSAP. *Mol Cell Biol.* **12**, 2662-2672.

Krauss, S., Johansen, T., Korzh, V. and Fjose, A. (1991a). Expression pattern of zebrafish *pax* genes suggests a role in early brain regionalization. *Nature* **353**, 267-270.

Krauss, S., Johansen, T., Korzh, V., Moens, U., Ericson, J. U. and Fjose, A. (1991b). Zebrafish *pax*[*zf-a*]: a paired box-containing gene expressed in the neural tube. *EMBO J.* **10**, 3609-3619.

Krauss, S., Johansen, T., Korzh, V. and Fjose, A. (1991c). Expression of the zebrafish paired box gene *pax*[*zf-b*] during early neurogenesis. *Development* **113**, 1193-206

Kuhlenbeck, H. (1973). *The Central Nervous System of Vertebrates. Vol. 3, part II. Overall Morphologic Pattern.* S. Karger, Basel.

Kuratani, S. C. (1991). Alternate expression of the HNK-1 epitope in rhmombomeres of the chick embryo. *Dev. Biol.* **144**, 215-219.

Lakke, E. A. J. F., van der Veeken, J. G. P. M., Mentink, M. M. T. and Marani, E. (1988). A SEM study on the development of the centricular surface morphology in the diencephalon of the rat. *Anat Embryol.* **179**, 73-80.

LaMantia, A.-S., Colbert, M. and Linney, E. (1993). Retinoic acid induction and regional differentiation prefigure olfactory pathway formation in the mammalian forebrain. *Neuron* **10**, 1035-1048.

Lamb, T. M., Knecht, A. K., Smith, W. C., Stachel, S. E., Economides, A. N., Stahl, N., Yancopolous, G. D. and Harland, R. M. (1993). Neural induction by the secreted polypeptide noggin. *Science* **262**,713-718.

Land, M. F. (1992). The evolution of eyes. *Annu. Rev. Neurosci.* **15**, 1-29.

Layer, P. G. and Alber, R. (1990). Patterning of chick brain vesicles as revealed by peanut agglutinin and cholinesterases. *Development* **109**, 613-624.

Lee, H. -C. and Auresperg, N. (1980). Calcium in epithelial cell contraction. *J. Cell Biol.* **85**, 325-336.

Legouis, R., Hardelin, J.-P., Levilliers, J., Claverie, J.-M., Compain, S., Wunderle, V., Millasseau., P., Le Pasiler. D., Cohen, D., Caterina, D., Bougueleret, L., Delemarre-Van de Waal, H., Lutfalla, G., Weissenbach, J. and Petit, C. (1991). The candidate gene for the X-linked Kallmann syndrome encodes a protein related to adhesion molecules. *Cell* **67**, 423-435.

Leussink, J. A. (1970). The spatial distribution of inductive capacities in the neural plate and archenteron roof of urodeles. *Netherlands J. Zool.* 20, 1-79.

Li, H.-S., Yang J.-M., Jacobson R.D., Pasko D., and Sundin O. (1994). *Pax-6* is first expressed in a region of ectoderm anterior to the early neural plate: implications for stepwise determination of the lens. Dev. Biol. **162** 181-194.

Liao, F., Giannini, S. L. and Birshtein, B. K. (1992). A nuclear DNA binding protein expressed during early stages of B cell differentaition interacts with diverse elements within the 3' end of the IgH chain gene cluster. *J. Immunol.* **148**, 29090-2917.

Liedke, K. B. (1955). Studies on lens induction in *Amblystoma punctatum. J. Exp. Zool.* **130**, 353-379.

Liesi, P. and Silver, J. (1988). Is astrocyte laminin involved in axon guidance in the mammalian CNS? *Dev. Biol.* **130**, 744-785.

Lindsell, C. E., Shawber, C. J., Boulter, J. and Weinmaster, G. (1995). Jagged: a mammalian ligand that activates Notch1. *Cell* **80**, 909-917.

Livne, I. Gibson, M. J. and Silverman, A. J. (1992). Gonadatrophin releasing hormone (GnRH) neurons migrate from olfactory placode through nasal septum, into forebrain. *Brain Res. Dev. Brain Res.* **69**, 117-123.

Lois, C. and Alvarez -Buylla, A.(1994). Long-distace neuronal migration in the adult mammalian brain. *Science* **264**, 1145-1148.

Lo Turco, J. J. and Kriegstein, A. R. (1991). Clusters of coupled neuroblasts in embryonic neocortex. *Science* **252**, 563-566.

Lumsden, A. and Guthrie, S. (1991). Alternating patterns of cell surface properties and neural crest cell migration suring segmentation of the chick hindbrain. *Development Suppl.* **2**, 9-15.

Lumsden, A. and Keynes, R. (1989). Segmental patterns of neuronal development in the chick hindbrain. *Nature* 337, 424-428.

Lumsden, A., Sprawson, N. and Graham, A. (1991). Segmental origin and migration of neural crest cells in the hindbrain region of the chick embryo. *Development* **113**, 1281-1291.

Luskin, M. B., Pearlman, A. L. and Sanes, J. R. (1988). Cell lineage in the cerebral cortex of the mouse studied in vivo and in vitro withj a recombinant retrovirus. *Neuron* **1**, 635-647.

Lutz, B., Kuratani, S., Rugarli, E. I., Wawersik, S., Wong, C., Bieber, F. R., Ballabio, A. and Eichele, G. (1994). Expression of the Kallmann syndrome gene in human fetal brain and in the manipulated chick embryo. *Hum. Mol. Genet.* **3**, 1717-1723.

Lyon, M. F. and Searle, A. G. (1989). *Genetic Variants and Strains of the Laboratory Mouse* , (2nd ed.). Oxford Univeristy Press. Oxford.

Macdonald, R., Xu, Q., Barth, K. A., Mikkola, I., Holder, N., Fjose, A., Krauss, S. and Wilson, S. W. (1994). Regulatory gene expression boundaries demarkate sites of neuronal differentiation in the embryonic zebrafish forebrain. *Neuron* **13**, 1039-1053.

Mackenzie, A., Leeming, G. L., Jowett. A. K., Ferguson, M. W. J. and Sharpe, P. T. (1991a). The homeobox gene *Hox 7.1* has specific region and temporal expression patterns during early murine craniofacial embryogenesis, especially tooth development *in vivo* and *in vitro*. *Development* **111**, 269-285.

Mackenzie, A., Ferguson, M. W. J. and Sharpe, P. T. (1991b). *Hox-7* expression during murine craniofacial development. *Development* **113**, 601-611.

Marin, F. and Puelles, L. (1994). Patterning of the embryonic avian midbrain after experimental inversions: A polarizing activity from the isthmus. *Dev. Biol.* **163**, 19-37.

Marin-Padilla, M and Amieva, M. R. (1989). Early neurogenesis of the mouse olfactory nerve: golgi and electron microscopic studies. *J. Comp. Neurol.* **288**, 339-352

Marsh-Armstrong, N., McCaffery, P., Gilbert, W., Dowling, J. E. and Dräger, U. C. (1994). Retinoic acid is necessary for development of the ventral retina in zebrafish. *Proc. Natl. Acad. Sci. USA* **91**, 7286-7290.

Martin P., Carriere C., Dozier D., Quatannens B.,Mirabel M.A., Vandenbunder B., Stehelin D., and Saule S. (1992). Characterization of a paired box- and homeobox-containing quail gene (*Pax-QNR*) expressed in the neuroretina.*Oncogene* **7** 1721-1728.

Martinez, S. and Alvarado-Mallart, R. -M. (1990). Expression of the homeobox *Chick-en* gene in chick/quail chimeras with inverted mes-metencephalic gratfs. *Dev. Biol.* **139**,432-436.

Matsuo, T., Osumi-Yamashita, N., Noji, S., Ohuchi, H., Koyama, E., Myokai, F., Matsuo N., Taniguchi S., Doi H., Iseki S., Nimomiya Y., Fujiwara M., Watanabe T. and Eto K. (1993). A mutation in the *Pax-6* gene in rat *small eye* is associated with impaired migration of midbrain neual crest. *Nature Genet.* **3**, 299-304.

Maulbecker C.C. and Gruss P. (1993). The oncogenic potential of *Pax* genes. *EMBO-J* **12** 2361-2367.

McCaffery, P., Lee, M. -O., Wagner, M. A., Sladek, N. E. and Dragler, U. C. (1992). Asymmetrical retinoic acid sysnthesis in the dorsoventral axis of the retina. *Development* **115**, 371-382.

McConnell, S. (1988). Fates of visual cortical neutons in the ferret after isochronic and heterochronic transplantation. *J. Neurosci.* **8**, 945-974.

McConnell, S. K. and Kaznowski, C. E. (1991) Cell cycle dependence of laminar determination in developing neocortex. *Science* **254**, 282-285.

McGinnis, W. and Krumlauf, R. (1992). Homeobox genes and axial patterning. *Cell* **68**, 283-302.

Moase, C. E. and Trasler, D. G. (1989). Spinal ganglia reduction in the *Splotch-delayed* mouse neural tube defect mutant. *Teratology* **40**, 67-75.

Moase, C. E. and Trasler, D. G. (1990). Delayed neural crest emigration from *Sp* and Sp^{d} mouse neural tube explants. *Teratology* **42**, 171-182.

Moase, C. E. and Trasler, D. G. (1991). N-CAM alterations in splotch neural tube defect mouse embryos. *Development* **113**, 1049-1058.

Moline, M. L. and Sandlin, C. (1993). Waardenburg syndrome and meningomyelocele. *Am J. Hum. Genet.* **47**, 126.

Monaghan, A. P., Davidson, D. R., Sime, C., Graham, E., Baldock, R., Bhattacharya, S. S. and Hill, R. E. (1991). The *Msh*-like homeobox genes define domains in the developing vertebrate eye. *Development* **112**, 1053-1061.

Monaghan., A. P., Kaestner. K. H., Grau, E. and Schütz., G. (1993). Postimplantation expression patterns indicate a role for the mouse *forkhead*/HNF-3 α , β and γ genes in the determination of the definative endoderm, chordamesoderm and neuroectoderm. *Development* **119**, 567-578.

Morriss-Kay, G. M. (1981). Growth and development of pattern in the cranial neural epithelium of rat embryos during neurulation. *J. Embryol. Exp. Morph.* **65 Suppl.**, 225-241.

Murakami, S. and Arai, Y. (1994). Direct evidence of migration of LHRH neurons from the nasal region to the forebrain in the chick embryo: a carbocyanin dye analysis. *Neurosci. Res.* **19**, 331-338.

Narod, S. A., Siegel-Bartelt, J. and Hoffman, H. J. (1988). Cerebellar infarction in a patient with Waardenburg syndrome. *Am J. Med. Genet.* **31**, 903-907.

Naruse, I. and Keino, H. (1993). Induction of agenesis of the corpus callosum by the destruction of anlage of the olfactory bulb using fetal laser surgery *exo utero* in mice. *Dev. Brain. Res.* **71**, 69-74.

Neslon, L. B., Spaeth, G. L., Nowinski, T. S., Margo, C. E. and Jackson, L. (1984). Aniridia. A review. *Surv. Opthalmol.* **28**, 621-642.

Nornes, H. O., Dressler, G. L., Knapik, E. W., Deutsch, U. and Gruss, P. (1990). Spatially and temporally retricted expression of Pax-2 during murine neurogenesis. Development **109**, 797-809.

Nottebohm, E. Dambly-Chaudiere, C. and Grysen, A. (1992). The connectivity of chemosensory neurons is controlled by the gene poxn in *Drosophila. Nature* **359**, 829-831.

Nottebohm, E., Usui A., Therianos, S., Kimura, K. -I, Dhambly-Chaudiere, C. and Ghysen, A. (1994) The poxn gene controls different steps of the formation of chemosensory organs in *Drosophila. Neuron*, **12**, 25-34

Nüsslein-Vohard, C. and Wieschaus, E. (1980). Mutations affecting segment number and polarity in *Drosophila. Nature* **287**, 795-801

Okada, T. S. (1991). *Transdifferentiation: flexibility in cell differentiation.* Oxford University Press, Oxford.

O'Rouke, N. A., Dailey, M. E., Smith, S. J. and McConnell, S. K. (1992). Diverse migratory pathways in the developing cerebral cortex. *Science* **258**, 299-302.

Ordahl, C. P. and Le Douarin, N. M. (1992). Two myogenic lineages within the developing somite. *Development* **114**, 339-53.

Osumi-Yamashita, N., Ninomiya, Y., Doi, H. and Eto, K. (1994). The contribution of both forebrain and midbrain crest cells to the mesenchyme in the frontonasal mass of mouse embryos. *Dev. Biol.* **164**, 409-419.

Palmatier, B. Y and Hilfer, S. R. (1977). Control of shape changes during early thyroid and optic cup development. *J. Cell. Biol.* **75**, 252a.

Pantke, O. A. and Cohen, M. M. Jr. (1971) The Waardenburg sysndrome. *Birth Defects: Original Article Series* Vol. **VII**(7) 147-152.

Patel, N. H. , Schafer, B., Goodman, C. S. and Holmgren, R. (1989). The role of segment polarity genes during *Drosophila* neurogenesis. *Genes Dev.* **3**, 890-904.

Pellier, V., Astic, L., Oestricher, A. B. and Saucier, D. (1994). B-50/GAP-43 expression by the olfactory receptor cells and neurons migrating from the olfactory placode in embryonic rats. *Dev. Brain. Res.* **80**, 63-72.

Peters, K. G., Werner, S., Chen, G. and Williams, L. T. (1992). Two FGF receptor genes are differentially expressed in epithelial and mesenchymal tissues during limb formation and organogenesis in the mouse. *Development* **114**, 233-43.

Peters, K., Ornitz, D., Werner, S. and Williams, L. (1993). Unique expression pattern of the FGF Receptor 3 gene during mouse organogenesis. *Dev. Biol.* **155**, 423-430.

Pexieder, T. and Jelinek, R. (1970). Pressure of the CSF and the morphogenesis of the CNS. Folia Morphol. **18**, 181-192.

Phelps, D. E. and Dressler, G. R. (1993). Aberrant expression of *Pax-2* in *Danforth's short tail*(*Sd*) mice. *Dev. Biol.* **157**. 251-258.

Pini, A. (1993). Chemorepulsion of axons in the developing central nervous system. *Science* **261**, 95-98.

Plachov, D., Chowdhury, K., Walther, C., Simon, D., Guenet, J -L. and Gruss, P. (1990). *Pax8*, a murine paired box gene expressed in the developing excretory system and thyroid gland. *Development* **110**, 643-651.

Placzek, M., Tessier-Lavinge, M., Yamada, T., Jessell, T. and Dodd, J. (1990). Mesodermal control of neural cell identity: Floor plate induction by the notochord. *Science* **250**, 985-988.

Placzek, M., Yamada, T., Tessier-Lavinge, M., Jessell, T. and Dodd, J. (1991). Control of dorsoventral pattern in vertebrate neural development: induction and polarizing properties of the floor plate. *Development Suppl.* **2**, 105-122.

Placzek, M., Jessell, T.M. and Dodd, J. (1993). Induction of floor plate differentiation by contact-dependent homeogenetic signals. *Development* **117**, 205-218.

Plaza, S., Dozier, C. and Saule, S. (1993). Quail *PAX-6* (*PAX-QNR*) encodes a transcription facor able to bind and trans-activate its own promoter. *Cell Growth Differ.* **4**, 1041-1050.

Price, M., Lemaistre, M., Pischetola, M., Di Lauro, R. and Dubule, D. (1991). A mouse gene related to *distal-less* shows a restricted expression in the developing nervous system. *Nature* **351**, 748-750

Price, M., Lazzaro, D., Pohl, T., Mattei, M. G., Ruther, U., Olivo, J. C., Duboule, D. and Di Lauro, R. (1992). Regional expression of the homeobox gene *Nkx-2.2* in the developing mammalian forebrain. *Neuron.* **8**, 241-255.

Poleev, A., Fickenscher, H., Mundlos, S., Winterpacht, A., Zabel, B., Fidler, A., Gruss, P. and Plachov, D. (1992). *PAX8*, a human paired box gene: isolation and expression in developing thyroid, kidney and Wilms' tumors. *Development* **116**, 611-623.

Ponte, I., Martinez, P., Ramirez, A., Jorcano, J. L., Monzo, M. and Suau, P. (1994). Transcriptional activation of Histone H1o during neuronal terminal differentiation. *Dev. Brain Res.* **80**, 35-44.

Puelles, L., Amat, J. A. and Martinez-De-La-Torre, M. (1987). Segment-related, maosaic neurogenic pattern in the forebrain and mesencephalon of early chick embryos: I. Topography of AChE-positive neuroblasts up to stages HH18. *J. Comp. Neurol.* **266**, 247-268.

Puelles, L. and Rubenstein, J. L. R. (1993). Expression of homeobox and other putative regulatory genes in the embryonic mouse forebrain suggests a neuromeric organization. *Trends Neurosci.* **16**, 472-479.

Puschel A.W., Gruss P., and Westerfield M. (1992). Sequence and expression pattern of *pax-6* are highly conserved between zebrafish and mice. *Development* **114** 643-651.

Quinlan, G. A., Williams, E. A., Tan. S.-S, and Tam. P. P. L. (1995). Neuroectodermal fate of epiblast cells in the distal region of the mouse egg cylinder: implication for body plan organization during early embryogenesis. *Development* **121**, 87-98.

Quiring, R., Walldorf, U., Kloter, U. and Gerhing, W. J. (1994). Homology of the *eyeless* gene of *Drosophila* to the *Small eye* gene in mice and *aniridia* in humans. Science 265, 785-789.

Rakic, P. (1988). Specification of cerebral cortical areas. *Science*, **241**, 170-176.

Ramon y Cajal, S. R. (1960). *Studies on Vertebrate Neurogenesis* (L. Guth trans.), C. C. Thomas, Spingfield, Illinois.

Read, A. P. (1995). Pax genes- Paired feet in three camps. *Nature Genetics* **9**, 333-334.

Reh T. A., Nagy, T. and Gretton, H. (1987). Retinal pigment epithelial cells induced to transdifferentiate to neurons by laminin. *Nature* **330**, 463-469.

Reh, T. A. and Tully, T. (1986). Regulation of tyrosine hydroxylase-containing amacrine cell number in larval frog retina. *Dev. Biol.* **114**, 463-469.

Ressler, K. J., Sullivan, S. L. and Buck, L. B. (1993). A zonal organisation of odorant receptor gene expression in the olfactory epithelium. *Cell* **73**, 597-609.

Richman, J. M. and Tickle, C. (1989). Epithelia are interchangeable between facial primordia of chick embryos and morphogenesis is controlled by the mesenchyme. *Dev. Biol.* **136**, 201-210.

Roberts, R. C. (1967). *Small eyes* - a new dominant eye mutant in the mouse. *Genet. Res. Camb.* **9**, 121-122.

Roelink, H., Augsburger, A., Heemskerk, J., Korzh, V., Norlin, S., Ruiz I Altaba, A., Tanabe, Y., Placzek, M., Edlund, T., Jessell, T. M. and Dodd, J. (1994). Floor plate and motor neuron induction by vhh, a vertebrate homolog of hedgehog expressed by the notochord. *Cell* **76**, 761-775.

Romanoff, A. L. (1960). *The Avian Embryo, Structure and Functional Development.* Macmillan, New York

Rothenpieler, U. W. and Dressler, G. R. (1993). Pax-2 is required for mesenchyme-to-epithelium conversion during kidney development. *Development* **119**, 711-720.

Rothman, P., Li, S. C., Gorham, B., Glimcher, L., Alt, F. and Boothby, M. (1991). Identification of a conserved lipopolyscacharide-plus-interlukin-4-responsive element located at the promoter of the germ line epsilon transcript. *Mol. Cell Biol.* 11, 5551-5561.

Rugh, R. (1968). *The Mouse: Its Reproduction and Development.* Burgess Publishing Co. Reprinted 1990, Oxford University Press, Oxford.

Ruiz - Altaba, A. (1992). Planar and vertical signals in the induction and patterning of the *Xenopus* nervous system. *Development* **115**, 67-80.

Ruiz - Altaba, A. (1993). Induction and axial patterning of the neural plate: planar and vertical signals. *J. Neurobiol.* **24**, 1276-1304.

Ryan, G., Steel-Perkins, V., Morris, J. F., Rauscher, F. J. III, and Dressler, G. R. (1995). Repression of Pax-2 by WT1 during normal kidney development. *Development* **121**, 867-875.

Saha, M. S., Spann, C. L. and Grainger, R.M. (1989) Embryonic lens induction: more than meets the optic vesicle *Cell. Differentiation and Development* **28**, 153-172.

Saha, M. S., Michel, R. B., Gulding, K. M. and Grainger, R. M. (1993). A *Xenopus* homeobox gene defines dorso-ventral domains in the developing brain. *Development* **118**, 193-202.

Salinas. P. C. and Nusse. R. (1992). Regional expression of the *Wnt-3* gene in the developing mouse forebrain in relationship to diencephalic neuromeres. *Mech. Dev.* **39**, 151-160.

Salser, S. J. and Kenyon, C. (1992). Activation of a *C. elegans Antennapedia* homologue in migrating cells controls their direction of migration. *Nature* **355**, 255-258.

Sander, K., Lohs-Schardin, M. and Baumann, M. (1980). Embryogenesis in a *Drosophila* mutant expressing half the normal segment number. *Nature* **287**, 841-843.

Sanyanusin, P., Schimmenti, L. A., McNoe, L. A., Ward, T. A., Pierpoint, M. E. M., Sullivan, M. J., Dobyns, W. B. and Eccles, M. R. (1995). Mutation of the *PAX2* gene in a family with optic nerve colobomas, renal anomalies and vesicoureral reflux. *Nature Genet.* **9**, 358-364.

Sasaki, H. and Hogan, B. L. (1993). Differential expression of multiple *forkhead* related genes during gastrulation and axial pattern formation in the mouse embryo. *Development* **118**, 47-59.

Schambra, U. B., Lauder, J. M. and Silver, J. (1992). *Atlas of The Prenatal Mouse Brain.* Academic Press, San Diego, CA.

Schlagger, B. L. and O'Leary, D. D. M. (1991). Potential of the visual cortex to develop an array of functional units unique to somatosensory cortex. *Science* **252**, 1556-1560.

Schmahl, W., Knoedlseder, M., Favor, J. and Davidson, D. (1993). Defects in neuronal migration and the pathogenesis of cortical malformations are associated with *Small eye (Sey)* in the mouse, a point mutation at the *Pax-6* locus. *Acta Neuropathol.* **86**, 126-135.

Schoenwolf, G. C. (1991). Cell movements driving neurulation in avian embryos. *Development Suppl.* **2**, 157-168.

Schoenwolf, G. C. and Smith, J. L. (1990). Mechanisms of neurulation: traditional viewpoint and recent advances. *Development* **109**, 243-270.

Schwanzel-Fukuda, M. and Pfaff, D. W. (1989). Origin of luteinizing hormone-releasing hormone neurons. *Nature* **338**, 161-164.

Schwanzel-Fukuda, M. and Pfaff, D. W. (1990). The migration of Luteinizing hormone-releasing hormone (LHRH)neurons from the medial nasal placode into the medial basal forebrain. *Experimentia* **46**, 956-962.

Schwanzel-Fukuda, M., Bick, D. and Pfaff, D. W. (1989). Luteinizing hormone-releasing hormone (LH-RH)-expressing cells do not migrate normally in an inherited hypogonadal (Kallmann) syndrome. *Mol. Brain Res.* **6**, 311-326.

Serafini, T., Kennedy, T. E., Galko, M. J., Mirzayan, C., Jessell, T. M. and Tessier-Lavigne, M. (1994). The netrins define a fmily of axon-outgrowth promoting proteins homologous to *C. elegans* UNC-6. *Cell* **78**, 409-424.

Serbedzija, G.N., Bronner-Fraser, M.and Fraser, S.E. (1992). Vital dye analysis of cranial neural crest cell migration in the mouse embryo. *Development* **116**, 297-307.

Servetnick, M. and Grainger, R. M.(1991.) Changes in neural and lens competence in *Xenopus* ectoderm: evidence for an autonomous developmental timer. *Development* **112**, 177-188.

Sharpe, C. R. and Gurdon, J. B. (1990). The induction of anterior and posterior neural genes in *Xenopus laevis*. *Development* **109**, 765-774.

Shawlot, W. and Behringer, R. R. (1995). Requirement for Lim1 in head-organizer function. *Nature* 374, 425-430.

Sheffield, J. B. (1982). Sorting behavior among cells from the embryonic chick neural retina. *Dev. Biol.* **89**, 41-47.

Shepherd, G. M. (1972). Synaptic organization of the mammalian olfactory bulb. *Physiol. Rev.* **52**, 864-917.

Sidman, R. L. (1961). Histogenesis of the mouse retina studied with thymidine-H^3. In "The Structure Of The Eye", (G. K. Smelser, Ed.). pp 487-506. Academic press, New York and London.

Silver, J. and Robb, R. M. (1979). Studies on the development of the eye cup and optic nerve in normal mice and in mutants with congenital optic nerve aplasia. *Dev Biol.* **68**,175-190.

Simeone, A., Guilsano, M., Acampora, D., Stornaiuolo, A., Rambaldi, M. and Boncinelli E. (1992). Two vertebrate homeobox genes related to the *Drosophila empty spiracles* gene are expressed in the embryonic cerebral cortex *EMBO J.* **11**, 2541-2550.

Simeone, A., D'Apice, M. R., Nigro, V., Casanova, J., Graziani, F., Acampora, D. and Avantaggiato, V. (1994). *Orthopedia*, a novel homeobox-containing gene expressed in the developing CNS of both mouse and *Drosophila*. *Neuron* **13**, 83-101.

Singh, M. and Birshtein, B. K. (1993). NF-HB (BSAP) is a repressor of the murine immunoglobulin heavy-chain 3' α enhancer at early stages of B-cell differentiation. *Mol-Cell-Biol.* **13**, 3611-22.

Smith, D. E. and Gridley, T. (1992). Differential screening of a PCR-generated mouse embryo cDNA library: glucose transporters are differentially expressed in early postimplantation mouse embryos. *Development* **116**, 555-561.

Soula, C., Foulquier, F., Duprat, A. M. and Cochard, P. (1993). Lineage analysis of early neural plate cells: cells with purely neuronal fate coexist with bipotential neuroglial precursors. *Dev. Biol.* **159**, 196-207.

Spemann, H. (1938). *Embryonic Development and Induction.* New Haven: Yale University Press.

Stapleton, P., Weith, A., Urbanek, P., Kozmik, Z. and Busslinger, M. (1993). Chromosomal localization of seven PAX genes and cloning of a novel family member, *PAX-9*. *Nature Genet.* **3**, 292-298.

Steel, K. P., Davidson, D. R., and Jackson, I. J. (1992). TRP-2/DT, a new early melanoblast marker shows that the steel growth factor (c-kit ligand) is a survival factor. *Development* **115**, 1111-1119.

Steindler, D. A., Cooper, N. G. F., Faissner, A. and Schachner, M. (1989). Boundaries defined by adhesion molecules during devlopment of the cerebral cortex: The J1:tenascin glycoprotein in the mouse somatosensory cortical barrel field. *Dev. Biol.* **131**, 243-260.

Storey, K. G., Crossley, J. M., De Robertis, E. M., Norris, W. E. and Stern, C. D. (1992). Neural induction and regionalisation in the chick embryo. *Development* **114**, 729-741.

Stoykova A. and Gruss, P. (1994). Roles of *Pax*-genes in the developing and adult brain as suggested by expression patterns. *J. Neurosci.* **14**, 1395-1412.

Stout, R. P. and Graziadei, P. P. C (1980). Influence of the olfactory placode on the development of the brain in *Xenopus laevis* (Daudin) I. Axonal growth and connections of the transplanted olfactory placode. *Neuroscience* **5**, 2175-2186.

Stuart, E. T., Kioussi, C. and Gruss, P. (1994). Mammalian Pax genes. *Annu. Rev. Genet.* **28**, 219-236.

Sulik, K. K., Cook, C. S. and Webster, W. S. (1988). Teratogens and craniofacial malformations: relationships to cell death. *Development* **103 Suppl.**, 213-232.

Svoboa, K. K. H. and O'Shea, K. S. (1987). An analysis of cell shape and the neuroepithelial basal laminar during optic vesicle formation in the mouse. *Development* **100**, 185-200.

Takada, S., Stark, K. L., Shea, M. J., Vassileva G., McMahon, J. A., and McMahon, A. P. (1994). *Wnt-3a* regulates somite and tailbud formation in the mouse embryo. *Genes. Dev.* **8**, 174-189.

Tan, S.-S. and Breen, S. (1993). Radial mosaicism and tangential cell dispersion both contribute to mouse neocortical development. *Nature*, **362**, 638-640.

Tao, W. and Lai, E. (1992). Telencephalon restricted expression of *BF-1*, a new member of the HNF-3/*forkhead* gene family, in the developing brain. *Neuron* **8**, 957-966.

Tassabehji, M., Read, A. P., Newton, V. E., Harris, R., Balling, R., Gruss, P. and Strachan, T. (1992). Waardenburg's syndrome patients have mutations in the human homologue of the *Pax-3* paired box gene. *Nature* **355**, 635-636.

Tessier-Lavinge, M., Placzek, M., Dodd, J. and Jessell, T. M. (1988). Chemotropic guidance of developing axons in the mammalian central nervous system. *Nature* **336**, 775-778.

Theiler, K. (1989). *The House Mouse :Atlas of Embryonic Development.* Springer-Verlag, New York, Berlin, Heidelberg.

Thomas, T. and Dziadek, M. (1993). Capacity to form choroid plexus-like cells in vitro is restricted to specific regions of the mouse neural ectoderm. *Development* **117**, 253-262.

Timmons, P. M., Wallin, J., Rigby, P. W. J. and Balling, R. (1994). Expression and function of *Pax1* during development of the pectoral girdle. *Development* **120**, 2773-2785

Ton, C. C. T., Hirovenen, H., Miwa, H., Weil, M.W., Monaghan, A. P., Jordan, T., van Heyningen, V., Hastie, N. D., Meijers-Heijboer, H., Drechsler. M,. Royer-Pokora, B., Collins, F., Swaroop, A., Strong, L. C. and Saunders, G. F. (1991). Positional cloning and characterization of a paired box and homeobox containing gene from the aniridia region.*Cell* **67**, 1059-1074.

Travis, J. (1994). Axon guidance: Wiring the nervous system. *Science* **266**, 568-570.

Treisman, J., Harris, E. and Desplan, C. (1991). The paired box encodes a second DNA-binding domain in the Paired homeodomain protein. *Genes Dev.* **5**, 594-604.

Tsukamoto, K., Nakamura, Y. and Niikawa, N. (1994). Isolation of two isoforms of the PAX3 gene transcripts and their tissue-specific alternative expression in human adult tissues. *Hum. Genet.* **93**, 270-274.

Turner, D. L. and Cepko, C. L. (1987). A common progenitor for neurons and glia persists in rat retina late in development. *Nature* **328**, 131-136

Turner, D. L., Snyder, E. Y. and Cepko, C. L. (1990). Lineage-independent determination of cell type in the embryonic mouse retina. *Neuron* **4**, 833-845.

Urbanek, P., Wang, Z.-Q., Fetka, I., Wagner, E. F. and Busslinger, M. (1994). Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP. Cell 79, 901-912.

Valverde, F. (1965). *Studies on The Piriform Lobe*. Harvard University Press, Cambridge MA.USA.

van Straaten, H. M. W., Hekking, J. W. M., Wiertz-Hoessels, E. L., Thors, F. and Drukker, J. (1988). Effect of the notochord on the differentiation of a floor plate area in the neural tube of the chick embryo. *Anat. Embryol.* **177**, 317-324.

Vasser, R., Chao, S. K., Sitchmeran, R., Nuñez, J. M., Vosshall, L. B. and Axel, R. (1994). Topographic organisation of sensory projections to the olfactory bulb. *Cell* **79**, 981-991.

Vogan, K. J., Epstein, D. J., Trasler, D. G. and Gros, P. (1993). The *splotch-delayed* (*Sp*^{d}) mouse mutant carries a point mutation within the *paired* box of the *Pax-3* gene. *Genomics* **17**, 364-9.

Vortkamp, A., Gessler, M. and Grzeschik, K-H. (1991). *GLI3* zinc finger gene interupted by translocations in Greig syndrome families. *Nature* **352**, 539-540.

Vortkamp, A., Franz, T., Gessler, M. and Grzeschik, K-H. (1992). Deletion of *GLI3* supports the homology of the human Greig cephalopolysyndactyly syndrome (GCPS) and the mouse mutant *extra toes* (*Xt*). *Mammalian Genome* **3**,461-463.

Waddington, C. H. (1932). Experiments on the development of the chick and the duck embryo cultivated in vitro. *Proc. Trans R. Soc. Lond. (B)* **211**, 179-230.

Waddington, C. H. and Cohen, A. (1936). Experiments on the devlopment of the head of the chick embryo. *J. Exp. Biol.* **13**, 219-236.

Wagner, M., Thaller, C., Jessell, T. and Eichele, G. (1990). Polarizing activity and retinoid snythesis in the floor plate of the neural tube. *Nature* **345**, 819-822.

Wakatsuki, Y. W., Neurath, M. F., Max, E. E. and Strober, W. (1994). The B-cell specific transcription factor BSAP regulates B cell proliferation . *J. Exp. Med.* **179**, 1099-1108.

Waid, D. K. and McLoon, S. C. (1995). Immediate differentiation of ganglion cells following mitosis in the developing retina. *Neuron* **14**, 117-124.

Wallin, J., Wilting, J., Koseki, H., Fritsch, R., Christ, B. and Balling, R. (1994). The role of *Pax-1* in axial skeleton development. *Development* **120**, 1109-1121.

Walsh, C. and Cepko, C. L. (1992). Widespread dispersion of neuronal clones across functional regions of the cerebral cortex. *Science* **255**, 434-440.

Walsh, C. and Cepko, C. L. (1993). Clonal dispersion in proliferative layers of developing cerebral cortex. *Nature* **362**, 632-635.

Walther C. and Gruss P. (1991). *Pax-6*, a murine paired box gene , is expressed in the developing CNS. *Development* **113**,1435-1449.

Walther C., Guenet J.L., Simon D., Deutsch U., Jostes B., Goulding M.D., Plachov D., Balling R., and Gruss P. (1991). *Pax*: a murine mutigene family of paired box-containing genes. *Genomics* **11**,424-434.

Watanabe, K., Araki, M., and Iwasaki, H. (1992). The embryonic pineal body as a multipotent organ. *Microscopy Research and Technique* **21**, 218-226.

Waters, S. J., Saikh, K. U. and Stavnezer, J. (1989). A B cell -specific nuclear protein that binds to DNA sites 5' to immunoglobulin S alpha tandem repeats is regulated during differentiation. *Mol. Cell Biol.* **9**, 5594-5601.

Watterson, R. L. (1965). Structure and Mitotic Behavior of the Early Neural Tube. *In* "Organogenesis" (R. L. DeHaan and H. Ursprung, Eds.), pp129-159. Holt, Rinehart and Winston, New York.

Weinstein, D. C., Ruiz-i-Altaba, A., Chen, W. S., Hoodless, P., Prezioso, V. R., Jessell, T. M. and Darnell J. E. Jr.(1994). The winged-helix transcription factor *HNF-3* β is required for notochord development in the mouse embryo. *Cell* **78**, 575-588.

Wilkinson D. G. (1992). Whole mount *in-situ* hybridization of vertebrate embryos. *In*, "*In-situ* hybridization: A practical approach" (D. G. Wilkinson, Ed.). pp 75-83. IRL Press, Oxford.

Wilson, D. (1974). Proliferation in the neural tube of the *Splotch* (*Sp*) mutant mouse. *J. Comp. Neurol.* **154**, 249-256.

Wilson, S. W., Ross, L. A., Parrett, T. and Easter, S. E. Jr. (1990). The development of a simple scaffold of axon tracts in the brain of the embryonic zebrafish, *Brachydanio rerio*. *Development* **108**, 121-145.

Xu, L., Kim, M. G. and Marcu, K. B. (1992a). Properties of B cell stage specific and ubiquitous nuclear factors binding to immunoglobulin heavy chain gene switch regions. *Int. Immunol.* **4**, 875-887.

Xu, Y., Baldassare, M., Fisher, P., Rathburn, G., Oltz, E. M., Yancopoulos, G. D., Jessel, T. M. and Alt, F. W. (1992b). *LH-2*: a LIM/homeodomain gene expressed in developing lymphocytes and neural cells. *Proc. Natl. Acad. Sci. USA* **90**, 227-231.

Yamada, T., Pfaff, S. L., Edlund, T. and Jessell, T. M. (1993). Control of cell pattern in the neural yube: motor neuron induction by diffusible factors from the notochord and floor plate. *Cell* **73**, 673-686.

Yamamoto, Y. (1976). Growth of lens and ocular environment: Role of neural retina in the growth of mouse lens as revealed by implantation experiment. *Dev. Growth Diff.* **18**, 273-278.

Yang, J.-J. and Hilfer, S.R. (1982). The effect of inhibitors of glycoconjugate synthesis on optic cup formation in the chick embryo. *Dev. Biol.* **92**, 41-53.

Young, R. W. (1985). Cell proliferation during postnatal development of the retina in the mouse. *Dev. Brain. Res.* **21**. 229-239.

Yu, R. T., McKeown, M., Evans, R. M. and Umesono K. (1994). Relationship between *Drosophila* gap gene *tailless* and a vertebrate nuclear receptor, Tlx. *Nature* **370**, 375-379.

Zannini M, Francis-Lang, H. Plachov D, Di Lauro R. (1992). Pax-8, a paired domain containing protein, binds to a sequence overlapping the recognition site of a homeodomain and activates transcription from two thyroid-specific promoters. *Mol. Cell Biol.* **12** 4230-4241

Zhang, Y., Ungar, A., Fresquez, C. and Holmgren, R. (1994). Ectopic expression of either the *Drosophila gooseberry*-distal or proximal gene causes alterations of cell fate in the epidermis and central nervous system. *Development* **120**, 1151-1161.

Zimmer, A. and Zimmer, A. (1992). Induction of a *RARβ2-lacZ* transgene by retinoic acid reflects the neuromeric organisation of the central nervous system. *Development* **116**, 977-983.

Zwaan, J. (1983). The appearance of alpha-crystallin in relation to cell cycle phase in the embryonic mouse lens. *Dev. Biol.* **96**, 173-181.

Zwilling, E. (1934) Induction of the olfactory placode by the forebrain in *Rana pipiens*. *Proc. Soc. Exp. Biol.* **31**, 933-935.

Zwilling, E. (1940) An experimental analysis of the development of anuran olfactory organ. *J. Exp. Zool.* **84**, 291-323.

APPENDIX

Previously published work included in this thesis

Development 121, 1433-1442 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

The role of *Pax-6* in eye and nasal development

Justin C. Grindley, Duncan R. Davidson and Robert E. Hill

Developmental Genetics Section, MRC Human Genetics Unit, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, UK

SUMMARY

***Small eye* (*Sey*) mice homozygous for mutations in the *Pax-6* gene have no lenses and no nasal cavities. We have examined the ontogeny of eye and nasal defects in *Sey/Sey* embryos and have related the defects seen to the pattern of *Pax-6* mRNA expression in the mouse during normal eye and nasal development.**

There are two principal components of the early eye, the neural ectoderm of the optic vesicle, which forms the retina, and the overlying surface ectoderm, which forms the lens and cornea. By studying these interacting tissues in normal and *Sey/Sey* embryos, we have identified processes for which Pax-6 is important and can thus suggest possible roles for the *Pax-6* gene.

Pax-6 is essential for the formation of lens placodes from surface ectoderm. In normal development, early *Pax-6* mRNA expression in a broad domain of surface ectoderm is downregulated, but expression is specifically maintained in the developing lens placode. Moreover, other *Pax-6*-expressing tissues are frequently those that have can trans-differentiate into lens. Thus, phenotype and expression together suggest a role for Pax-6 in lens determination.

At least some functions of Pax-6 can be separated from the influence of other tissues. Early *Sey/Sey* optic vesicles are abnormally broad and fail to constrict proximally. These defects occur prior to the time of lens placode formation and probably reflect a requirement for Pax-6 in neural ectoderm. In surface ectoderm domains, where *Pax-6* expression is known to be independent of the presence of an optic vesicle, Pax-6 function is required for the maintenance of its own transcription.

The mutual dependency of lens and optic vesicle development can also be studied using the *Small eye* mutation. Using region-specific markers we find that, in the morphologically abnormal *Sey/Sey* optic vesicles, aspects of normal proximo-distal specification nevertheless persist, despite the complete absence of lens.

Like the lens, the nasal cavities develop from ectodermal placodes that normally express *Pax-6* mRNA, fail to form in *Sey/Sey* mice and show Pax-6-dependent *Pax-6* mRNA regulation. Analysis of patterns of programmed cell death and absence of nasal region expression from an *Msx-1* transgene in *Sey/Sey* embryos suggest a requirement for Pax-6 in the transition from presumptive nasal ectoderm to placode, and that *Msx-1*, or genes regulating it, are possible targets for Pax-6.

Key words: *Pax-6*, *Small eye*, craniofacial development, optic vesicle, nasal placode, lens placode, mouse

INTRODUCTION

Pax-6, independently isolated by homology to *gooseberry distal* (Walther and Gruss, 1991), and from positional cloning at the aniridia locus (Ton et al., 1991), encodes two DNA-binding motifs, a paired domain (Bopp et al., 1986; Treisman et al., 1991) and a *paired*-like homeodomain (Frigerio et al., 1986).

Members of the Pax gene family (Walther et al., 1991), have regulatory roles during development (Chalepakis et al., 1991; Epstein et al., 1991; Baldwin et al., 1992; Tassabehji et al., 1992). A variety of anterior segment congenital eye abnormalities result from heterozygosity for mutations in the mouse, rat or human *Pax-6/PAX6* gene. These include mouse and rat *Small eye* (Hill et al., 1991; Matsuo et al., 1993), human Peters' anomaly (Hanson et al., 1994) and aniridia, congenital lack of iris (Jordan et al., 1992; Glaser et al., 1992; Hanson et al., 1993). These phenotypes can result from deletions encompassing the entire *Pax-6/PAX6* gene, suggesting that the mutations result in loss of protein function and that the phenotype is a result of haplo-insufficiency (Hill et al., 1991).

Small eye is a semidominant mutation. Homozygous mutant mice have no eyes and no nasal cavities. Since, like the lens, nasal cavities form from the invagination of an ectodermal placode, these phenotypes have been suggested by Hogan et al. (1986) to result from a failure of early placode differentiation. *Sey/Sey* embryos also have no olfactory bulbs (Hogan et al., 1986) and have defects in neuronal differentiation and migration that lead to abnormal cortical plate formation (Schmahl et al., 1993). *Pax-6* is thus necessary for the normal development of eyes, nose and brain. The range of abnormalities seen in *Sey* mice may indicate that *Pax-6* has a number of roles during normal development. We have concentrated our attention on the eye and nasal phenotypes.

MATERIALS AND METHODS

Embryonic material

We used non-pigmented Swiss mice as parents for all in situ hybridi-

sation experiments. Embryonic development is assumed to have begun at midnight of the night of mating. The allele of *Small eye* used is *Sey* (Lyon and Searle, 1989). Homozygous *Small eye* mice die at birth (Hogan et al., 1986), thus the *Sey/Sey* embryos used were obtained from crosses of heterozygous *Small eye* parents, which were selected on the basis of eye size. Littermates from these matings (*Sey*/+ and +/+ embryos) provided control embryos of matched developmental stage.

In those cases where we illustrate comparisons between *Sey/Sey* embryos and their littermates, we have also compared the littermates with wild-type embryos derived from wild-type parents. This was done using our own results and by reference to published data on patterns in normal mouse development for the markers that we have used (Steel et al., 1992; Walther and Gruss, 1991; Dong and Chung, 1991; Sulik et al., 1988).

Probes for in situ hybridisation

A gene-specific 1 kb template for making mouse *Pax-6* RNA probe was made by polymerase chain reaction, PCR, from a 1.6 kb cDNA clone, pm1 (Ton et al., 1991), using oligonucleotide C128 (ATGGTTTTCTAATCGAAGGG) from the 3′ end of the *Pax-6* homeobox, and a T7 promoter oligonucleotide (AATACGACTCACTATAG) matching vector sequence. A similar procedure was used to make an *entactin* probe: oligonucleotides D932 (CAGTGTCACCACAGCACTGGC) and T3 promoter oligonucleotide (ATTAACCCTCACTAAAG) were used to amplify a 1.6 kb region at the 3′ end of clone p633, a gift of Professor Albert E. Chung. PCR product was extracted by phenol-chloroform followed by ethanol precipitation before being transcribed. The template for the *Tyrp2* probe, a gift of Ian Jackson, was the 1.2 kb clone p5A7 described in Steel et al. (1992), digested with *Hin*dIII.

In situ hybridisation

Embryos were removed into ice-cold phosphate-buffered saline (PBS) and then fixed in 4% paraformaldehyde (PFA) in PBS at 4°C and processed routinely for histology. In situ hybridisation using ^{35}S-labelled riboprobe was performed as described in Monaghan et al. (1991). Control probes *Msx-1* and *Msx-2*, which detect transcripts of genes with known expression patterns, were used to identify non-specific signal. Where expression patterns in *Sey/Sey* and normal were compared, sections were hybridised in the same experiment, generally on the same slide. Autoradiographs using K5 Emulsion (Ilford), were exposed for 2.5 to 6 weeks and sections were stained with methyl green. Computer-aided overlaying of dark-field and bright-field images was performed as previously described (Monaghan et al., 1991).

PCR genotyping of young embryos

The *Sey* mutation (Hill et al., 1991) creates a diagnostic *Dde*I site that allows embryonic material to be genotyped by PCR amplification and *Dde*I digestion. For genotyping, paraformaldehyde-fixed embryos were bisected using tungsten needles in PBS at the level of the midgut (8 day) or heart (8.5 day and later embryos). Anterior portions were aligned a few millimetres apart and embedded in a 3% low melting point agarose gel made with PBS. The agarose block was cut in such a way that each embryo could later be unambiguously identified and then fixed in 4% PFA, processed routinely for histology and embedded in wax. In this way, similar sections of embryos of different genotypes appear on the same slide and are hybridised equivalently. Posterior embryo parts were individually washed twice in PBS then digested in 20 μl with 6 μg/ml proteinase K for 1 hour at 55°C in 1× PCR buffer, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin and 0.1% Triton X-100, with added non-ionic detergents, 0.4% Tween 20 and 0.4% NP40. Proteinase K was inactivated at 95°C for 10 minutes. 1-2 μl of digested sample was taken for each PCR reaction. PCR reactions were performed in total volumes of 25 μl using 1 pmole/reaction of each primer B509 (TGCCAGCAACAGGAAGGAGG) and E723 (CTTTCTCCAGAGCCTCAATCTG) with 0.2 mM of each dNTP in the 1× PCR buffer described above. Taq polymerase (1 unit/ reaction) was added after an initial denaturing step of 4 minutes at 95°C. 35 cycles (95°C for 30 seconds, 61°C for 30 seconds and 72°C for 48 seconds) were performed followed by a single extension step of 72°C for 5 minutes. Following phenol-chloroform extraction and ethanol precipitation, samples of PCR products were digested with *Dde*I. The 154 bp PCR product was cut to give 83 bp and 71 bp fragments for wild-type alleles and 83 bp, 51 bp and 20 bp fragments for *Sey* alleles. These digestion products were analysed on 4% NuSieve GTG agarose gels (FMC) in 1× TBE buffer.

Detection of cell death

We detected cell death using Nile Blue Sulphate staining (Sulik et al., 1988). Freshly dissected embryos were stained in 1:20,000 Nile Blue Sulphate in either PBS or PBT (PBS with 0.01% Tween 20) for 15 minutes and photographed immediately using 64T film on Wild M400 photomicroscope. The specificity of this procedure was confirmed by comparing the pattern of dark-staining cells with that generated using acridine orange DNA stain (Graham at al., 1993).

Msx-1 transgenic mice

Production of the transgenic mice carrying the reporter construct will be described elsewhere. Briefly the reporter construct contains 5 kb of sequence upstream of the mouse *Msx-1* gene and generates a fusion transcript with *lacZ* in frame with the *Msx-1* gene from an *Nco*I site 127 bp 3′ of the start of the *Msx-1* coding region. The construct uses SV40 poly(A) addition and transcription termination sequences. Transgenic mice were produced on a (CBA × C57BL/6)F_1 hybrid background. From matings of Swiss *Sey* mice to transgenics, *Sey* males carrying the transgene were identified by Southern hybridisation and PCR analysis. These were mated to *Sey* females. Embryos from these matings were obtained at E9.0 to E11.5 days and fixed in 2% formaldehyde, 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3), 2 mM $MgCl_2$, 5 mM EGTA, then stained for β-galactosidase activity (Beddington and Lawson, 1990). Fixation times were 10-30 minutes depending upon embryo size. Staining was for 5 hours in the dark at 37°C.

RESULTS

Optic vesicle development

Homozygous mutant *Sey/Sey* embryos can first readily be distinguished from littermates at about 9.5 days by the abnormal shape of the optic vesicle and forebrain (Fig. 1A,B). We have confirmed this morphological characterisation by PCR analysis of the genotype (see Materials and methods). The optic vesicle is constricted proximally in the wild-type embryo but not in the *Sey/Sey* embryo. This defect persists for some time: E10.5-E11.5 *Sey/Sey* embryos typically have a uniformly broad optic vesicle, distorted towards the distal end (Fig. 1D) giving the appearance of an abortive process of optic cup formation (Fig. 4A-C,E,G). In normal eye development, the optic vesicle interacts with the lens pit, growing around it to form a bilayered optic cup. The thin outer layer and thicker inner layer of the optic cup go on to form pigmented retinal epithelium and neural retina, respectively. Optic vesicles in E11.5-E15.5 *Sey/Sey* embryos display a bilayered neuroepithelial structure. The *Sey/Sey* optic stalk retains a lumen, unlike the normal optic nerve, which is a dense bundle of axons by E15.5.

Transmission EM analysis of *Sey/Sey* optic vesicles (data not shown) reveals that they make local contact with overlying

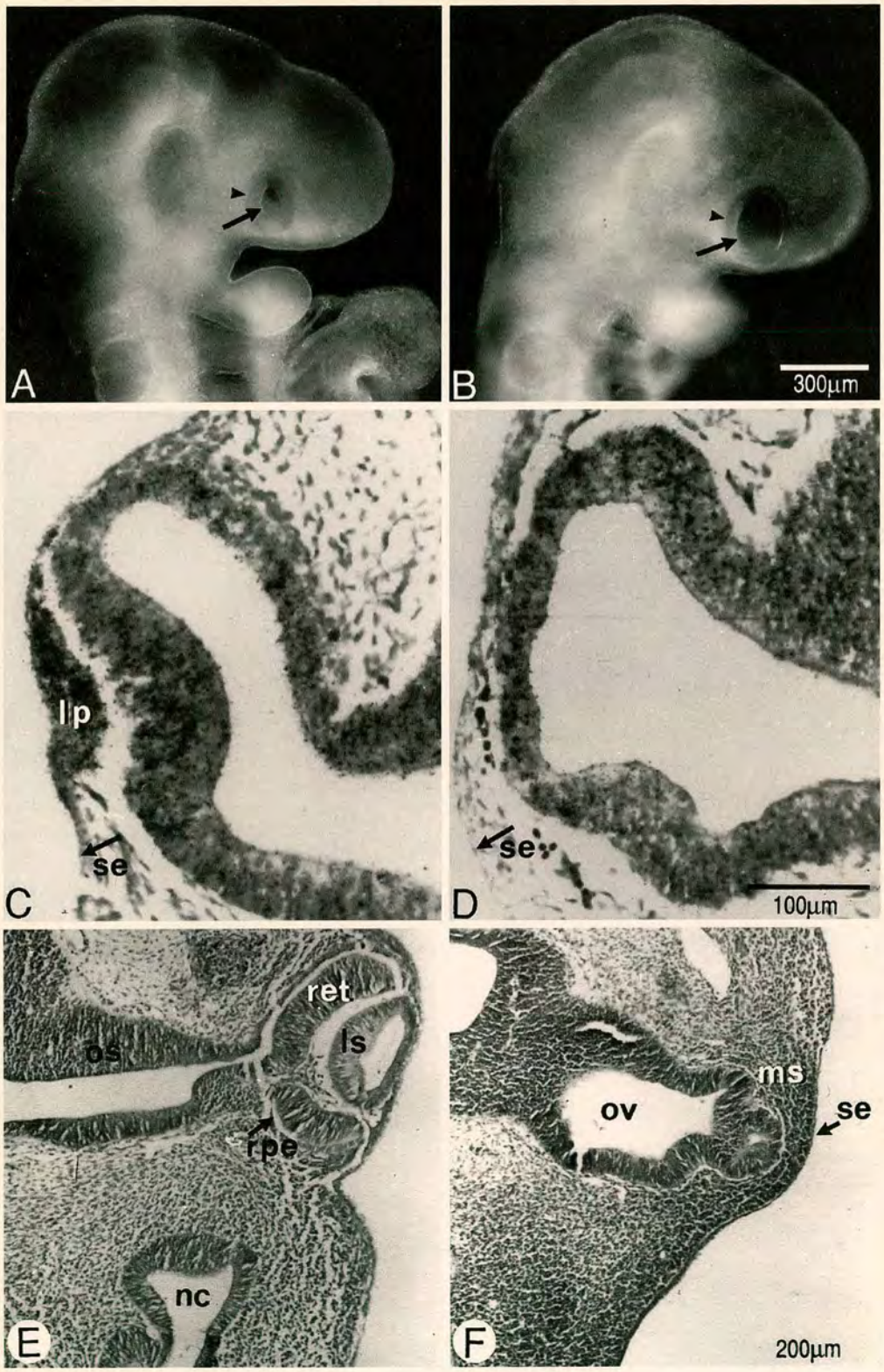

Fig. 1. Early phenotype of *Sey/Sey* embryos. Characteristic appearance of the eye at E9.5 of (A) wild-type embryo and (B) *Sey/Sey* embryo. *Sey/Sey* optic vesicle is broader than normal and has failed to constrict proximally. Arrows, proximal restriction of optic vesicles. Arrowheads, extent of the eye region. Histology of the eye at (C,D) E9.75 and (E,F) E11.5. At E9.75, the lens placode (lp), a prominent thickening of the surface ectoderm (se) is present in the littermate (C), but absent from the *Sey/Sey* embryo (D). Later *Sey/Sey* embryo, at E11.5 in F lacks the developing lens (ls) and nasal cavity (nc), present in littermate (E). Normal littermate optic vesicle has produced an optic stalk (os) and optic cup with distinct retina (ret), and pigmented retinal epithelium (rpe). *Sey/Sey* optic vesicle (ov), is broader than normal, is distorted at the distal end and is separated from surface ectoderm (se) by intervening mesenchymal-like cells (ms).

surface ectoderm at E9.5, but that this contact is progressively lost (shown at E15.5 in Fig. 4A) as mesenchymal cells intervene between the two tissues.

Absence of lens placodes in *Sey/Sey* embryos

Histological analysis of *Sey/Sey* embryos at E9.5 to E11.5 showed an absence of the thickened surface ectodermal component of the eye, the lens placode, lens pit and subsequent lens vesicle, which are present in their normal and *Sey*/+ littermates. At E9.75 when lens placodes are normally well developed (Fig. 1C), *Sey/Sey* embryos have no thickening of the surface ectoderm in the eye region (Fig. 1D). As a result,

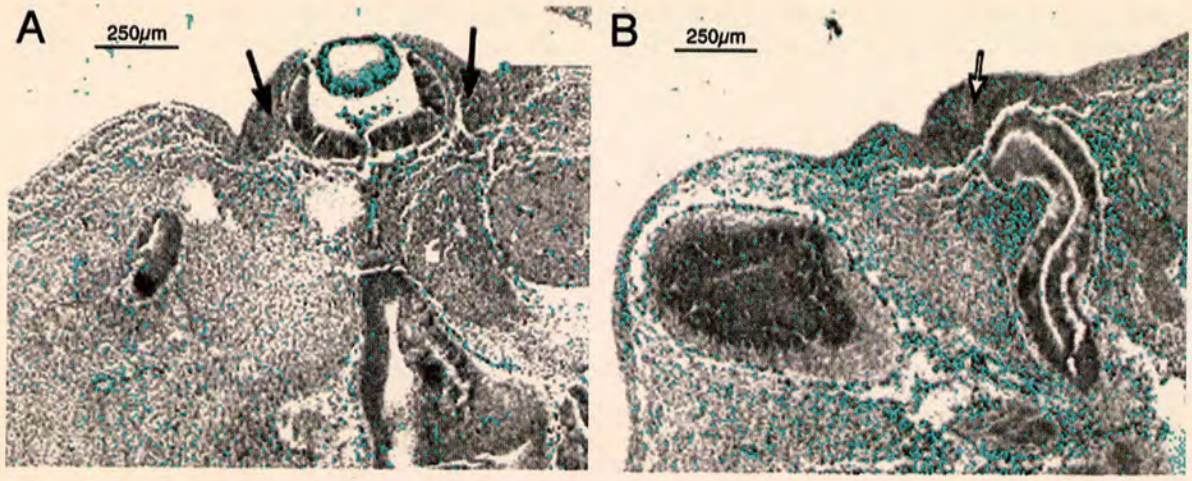

Fig. 2. Radioactive in situ hybridisation detecting expression of *entactin* mRNA, blue, in transverse sections at E12 of (B) *Sey/Sey* embryo and (A) normal littermate. Developing lens expresses high levels of *entactin* mRNA from E10 onwards, whereas condensing mesenchymal cells (arrows) around the optic cup in A have low levels of expression. A region of dense tissue (open arrow) present at the surface adjacent to the distal optic vesicle in B is typical for *Sey/Sey* embryos and has the appearance and expression characteristics of condensed mesenchyme rather than of developing lens. Anterior of sections is to the left.

there is no developing lens detectable in *Sey/Sey* embryos at E11.5 (Fig. 1F).

A region of dense tissue near the surface adjacent to the distal optic vesicle is typically present in homozygous *Small eye* mutants from E10 onwards. This is not abortive or delayed lens development, but is probably a condensation of mesenchymal cells. Developing lens (Fig. 2A) normally strongly expresses mRNA for the extracellular matrix protein gene *entactin* from the lens pit stage onwards (Dong and Chung, 1991). In contrast, the regions of dense tissue in *Sey/Sey* embryos express *entactin* mRNA at low levels (Fig. 2B) more comparable to the level of expression in mesenchymal cells that condense adjacent to the anterior optic cup in normal embryos. By high magnification microscopy, we confirmed that the regions of dense tissue seen at the surface in *Sey/Sey* mutants were condensations of mesenchymal-like cells under a non-placodal epithelial layer (data not shown).

For stages E12.5 onwards, when lens fiber differentiation could be detected in

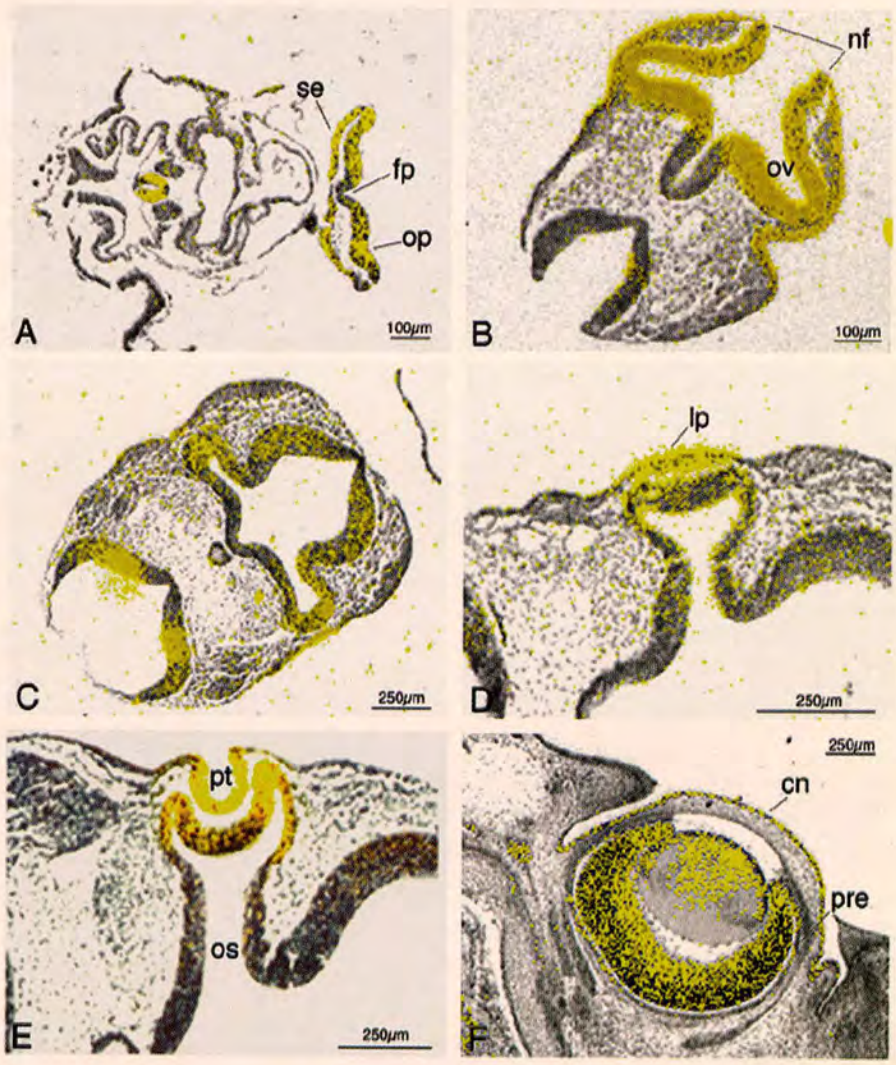

Fig. 3. Normal expression of *Pax-6* mRNA, yellow, during early eye development. Radioactive in situ hybridisation on transverse sections at embryonic ages (A) E8.0; (B) E8.5; (C) E9.25; (D) E9.5; (E) E10.5 and (F) E15.5. Surface ectoderm (se), optic pit (op), floorplate (fp), neural folds (nf),optic vesicle (ov), lens placode (lp), lens pit (pt), optic stalk (os), pigmented retinal epithelium (pre), cornea (cn).

controls, we found no evidence of lenses, lentoid bodies or of cells with the eosin-staining properties of lens fibers in any homozygous mutant examined.

Pax-6 expression in the neural ectoderm

We have examined *Pax-6* transcription in wild-type mice from 8 days to 15.5 days. At the earliest stage examined, E8.0, Pax-6 transcripts were detected over an extensive region of head neural ectoderm, including the optic pit, the first morphologically detectable indication of the eye region (Fig. 3A). There was no expression in the floorplate. At E8.5, shortly before headfold closure, much of the complexity of the later forebrain *Pax-6* expression pattern has been established. In the presumptive diencephalon, expression is mainly dorsal, reaching the neural folds, and is already segmented as has been reported for later stages (Walther and Gruss, 1991; Figdor and Stern, 1993; Puelles and Rubenstein, 1993). Transverse sections through the head at this stage typically show expression to be strong in the optic vesicle, weaker more rostrally in the presumptive telencephalon, with the neural ectoderm expression being undetectable or only weakly detectable at the neural folds (Fig. 3B).

Pax-6 expression within the optic vesicle is polarised distally. Thus, from E9.5 onwards expression in the optic vesicle is strongest around the rim of the developing optic cup (Fig. 3D) and is consistently weaker both in the back of the optic cup and in the proximal optic vesicle structures such as the developing optic stalk (Fig. 3E). In the early optic cup, *Pax-6* is expressed in both the prospective pigmented retinal epithelium (PRE) layer and prospective neural retina (Fig. 3E). This pattern is dynamic. Thus, by E15.5 expression within the PRE is only seen in anterior regions, i.e. near the rim of the optic cup. (Fig. 3F).

Restriction of *Pax-6* mRNA expression in surface ectoderm of wild-type mice

At E8.0, *Pax-6* mRNA is expressed in a broad region of head surface ectoderm covering the prosencephalon but not the hindbrain region or the presumptive first branchial arch (Fig. 3A). This expression in the surface ectoderm is still broad at E8.5 and extends rostrally to the neural folds. This is in contrast to the neural ectoderm expression at this stage, where strong expression is localised to the optic vesicle and presumptive diencephalon (Fig. 3B). Over the next 24 hours, expression in the surface ectoderm becomes restricted to the developing lens placode (Fig. 3D) nasal placode and immediately adjacent tissues. *Pax-6* mRNA expression in the lens placode forms part of a larger domain that extends in the dorsal-caudal direction (Fig. 3C). As lens pit formation proceeds, this expression domain is further restricted until it lies exclusively within the developing eye region (Fig. 3E). Similarly the expression in the ectoderm between the lens and nasal placodes persists longer than most non-placodal expression but, by E9.75, the lens and nasal placodes are clearly separated by a region of non-expressing ectoderm. Consistent with a previous report (Walther and Gruss, 1991), we find *Pax-6* mRNA expression continues in the parts of the eye derived from surface ectoderm until the last stage examined, E15.5 (Fig. 3F). This includes the lens pit, the lens vesicle and the lens as well as the developing cornea.

Pax-6 and *Tyrp2* expression domains in optic vesicles of *Sey/Sey* embryos

To characterise the structures formed by the optic vesicle in

Fig. 4. (A-C) Optic vesicle phenotype of *Sey/Sey* embryos at E15.5. (A) Transverse sections through optic vesicle (ov), showing separation from skin by intervening tissue. (B) Detail of bilayered optic cup structure (oc) and optic stalk (os). (C) *Pax-6* mRNA expression, predominantly in cup-like structure. (D-G) Expression domains of *Pax-6* mRNA (D,E) and *Tyrp2* mRNA (F,G) in transverse sections of control (D,F) and *Sey/Sey* (E,G) eyes at E12.5. Developing pigmented retinal epithelium (pre); developing neural retina (nr). Anterior is to the left.

the absence both of functional Pax-6 protein and of lens, we combined our histological analysis with in situ hybridisation using probes for gene transcripts that normally display region-specific expression patterns within the optic vesicle. The single base change in the *Sey* allele, whilst predicted to result in a truncated Pax-6 protein (Hill et al., 1991), will not affect the in situ hybridisation assay for *Pax-6* mRNA expression, thus Pax-6 can also be used as a marker in this way. We have also used the tyrosinase-related protein gene *Tyrp2* as an early optic vesicle marker that becomes restricted to RPE, the pigmented retinal epithelium layer of the optic cup, by about E10.5 (Steel et al., 1992). We examined *Sey/Sey* and control embryos hybridised with antisense probes for *Pax-6* (stages E8.0 to E15.5) or *Tyrp2* (stages E11.5-E12.5)

Pax-6 expression in *Sey/Sey* optic vesicles is initially indistinguishable from that in +/+ and *Sey*/+ littermates (Fig. 5B). As optic vesicle abnormalities become apparent in *Sey/Sey* mutants, *Pax-6* expression retains features seen in the expression patterns of littermates. Thus at E9.5 to E10.5, *Pax-6* expression is strongest in most distal structures (Fig. 5C). Later still, *Sey/Sey* optic vesicles form distinct optic stalk-like and bilayered optic-cup-like structures, shown at E15.5 in Fig. 4A,B. As for normal eyes, it is the two neuralectodermal layers of the optic-cup-like structure that express *Pax-6* mRNA (Fig. 4C) and there is little expression in the optic stalk.

Using the *Tyrp2* probe, there is typically a small domain of intense signal in the most distal regions of the *Sey/Sey* optic vesicle (Fig. 4G). Where the optic vesicle forms a cup, *Tyrp2* transcripts are most abundant at the rim of this cup, but are present in both layers. Unlike the clearly differentiated pigmented retinal epithelium and neural retina in the optic cups of normal embryos, the two neuroepithelial layers in those structures in *Sey/Sey* embryos that resemble cups are of similar thickness and appearance (Fig. 4E,G). In these structures, *Tyrp2* is almost exclusively expressed within the larger, but still distally restricted, *Pax-6* expression domains (Fig. 4E). This is in contrast to normal embryos, where there is a considerable region of pigmented retinal epithelium that expresses *Tyrp2* (Fig. 4F) but not *Pax-6* (Fig. 4D).

Surface ectoderm Pax-*6* mRNA expression in homozygous mutant mice

PCR genotyping (see Materials and methods) allows identification of *Sey/Sey* embryos prior to the appearance of morphologicaly recognisable defects. As mentioned above, the single base change in the *Sey* allele does not affect the in situ hybridisation assay for *Pax-6* mRNA expression, thus we have been able to analyse *Pax-6* expression in early *Sey/Sey* embryos.

Genotyped embryos were sectioned and hybridised with antisense *Pax-6* probe. *Sey/Sey* embryos showed no differences from the normal pattern of expression at E8.0 or E8.5 (Fig. 5A,B). By E9.5-E9.75, however, when in normal embryos expression was confined to around the lens and nasal placodes, we detected no expression in the surface ectoderm anywhere in the heads of homozygous mutants, including the eye region (Fig. 5C).

Pax-6 expression and nasal placode development

We found *Pax-6* mRNA (Fig. 6A) in nasal placode, though at a lower level than in lens placode. We find that this expression continues in placodal epithelium during the formation of nasal pits (Fig. 6B) and that, subsequently, expression is detectable in the developing olfactory epithelium, as has been previously reported by Walter and Gruss (1991). No nasal placodes could be detected on serial sections through the nasal region of *Sey/Sey* embryos at E9.75, whereas littermates at this stage have well-formed nasal placodes undergoing the first stages of invagination to form a nasal pit. The nasal placode *Pax-6* mRNA detectable in littermates (Fig. 6C) is absent from *Sey/Sey* embryos at this stage (Fig. 6D) although *Sey/Sey* embryos retain expression in the neuroepithelium of the developing forebrain.

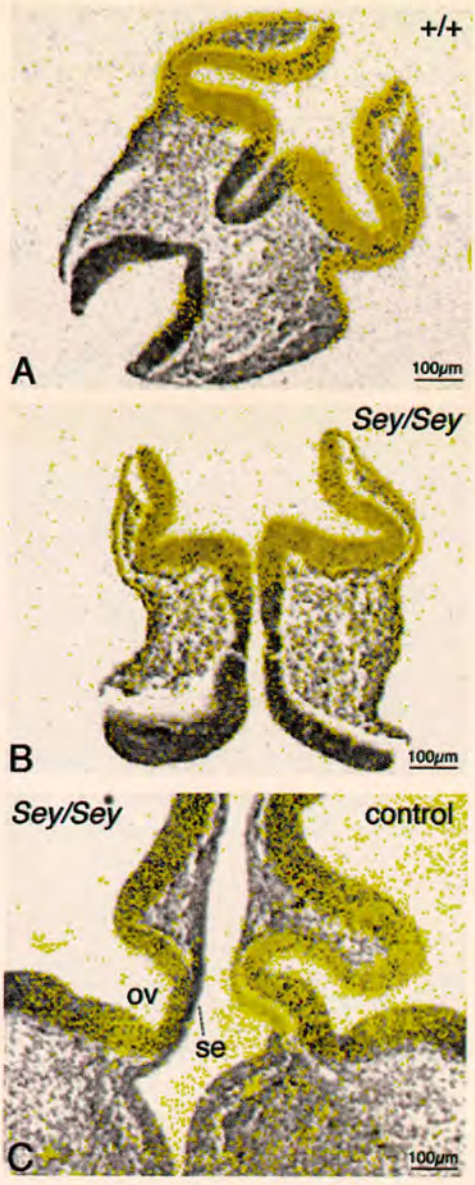

Fig. 5. *Pax-6* mRNA expression, yellow, for (A) wild-type embryo and (B) *Sey/Sey* embryo, at E8.5. *Sey/Sey* embryos at this stage have the same *Pax-6* mRNA expression pattern as their littermates. (C) By E9.75 surface ectoderm (se) expression adjacent to optic vesicle (ov) in littermate (right) is absent from *Sey/Sey* embryo (left).

Msx-1 transgene expression in the nasal placode region

In +/+ and *Sey*/+ mice carrying the the *Msx-1* transgene, lateral placodal epithelial cells express the *lacZ* marker at a high level, whereas cells in non-placodal ectoderm cells, underlying mesenchyme and medial nasal placode do not. This specific expression is detectable at E9.5 and develops into an arc of strong expression in the placodal ectoderm of the prospective lateral nasal process by E10.5 (Fig. 7A,B). X-gal staining of *Sey/Sey* embryos (Fig. 7C) and littermates (Fig. 7B) from E9.0 to E11.5 showed that this early marker for nasal placode differentiation is never expressed in the absence of functional Pax-6 protein.

Cell death in nasal placode region

In normal embryos, a narrow arc of cell death appears around the edge of the developing nasal placode prior to invagination (Fig. 7D). Patterns of cell death, detected by Nile Blue Sulphate (NBS) staining, can be used as markers to study the fate of cells in the nasal region in *Sey/Sey* embryos. In contrast to the restricted arcs of NBS-staining cells in normal embryos, we found diffuse patches of NBS-stained cells in the nasal region of E9.25-E9.75 *Sey/Sey* embryos (Fig. 7E). The number of stained cells varied considerably from embryo to embryo, and between left and right sides of the same embryo, but consistently these patches were diffuse. Analysing the cell death patterns at E10.5, we find that the majority of NBS-staining regions are identical in *Sey/Sey* embryos and their littermates, but there are three differences. (1) Cell death normally occurs at the point of fusion of the lateral and medial nasal processes. The position of the processes formed in *Sey/Sey* embryos and the cell death pattern on them identifies these as medial nasal processes, the cell death domains of the lateral nasal processes being absent. This supports the conclusions of previous scanning electron microscopy studies by Heinzmann et al. (1991) that the lateral nasal processes are absent in *Sey/Sey* embryos at this stage. (2) In 10.5 day *Sey/Sey* embryos, we observe bilateral ectopic bands of subectodermal NBS staining over the anterior forebrain. This may identify a population of midbrain neural crest cells that, in homozygous rat *Smalleye* (*rSey*) embryos, fail to reach the nasal region (Matsuo et al., 1993), and may die. (3) The normally narrow band of NBS staining along the midline of the brain is broadened in *Sey/Sey* embryos into a diamond shape patch over the telencephalon-diencephalon boundary.

DISCUSSION

Early optic vesicle abnormalities in *Sey/Sey* embryos

The optic vesicle and surface ectoderm both express *Pax-6* during normal development and both show abnormalities in *Sey/Sey* mice. In normal eye development, there are multiple interactions between these two tissues. The optic vesicle is thought to be important for correct positioning and growth of the lens (reviewed by Grainger, 1992). Equally, the developing lens appears to be important for growth of the retina. (Coulombre and Coulombre, 1964).

The first developmental defects of *Sey/Sey* embryos that we detect are the abnormal shape of the brain and optic vesicle. The failure of the optic vesicles to constrict proximally occurs before the time of normal lens development and may reflect a requirement for Pax-6 in the neural ectoderm. The subsequent absence of lens may contribute to the later distal distortion of *Sey/Sey* optic vesicles, but this phenotype might also result

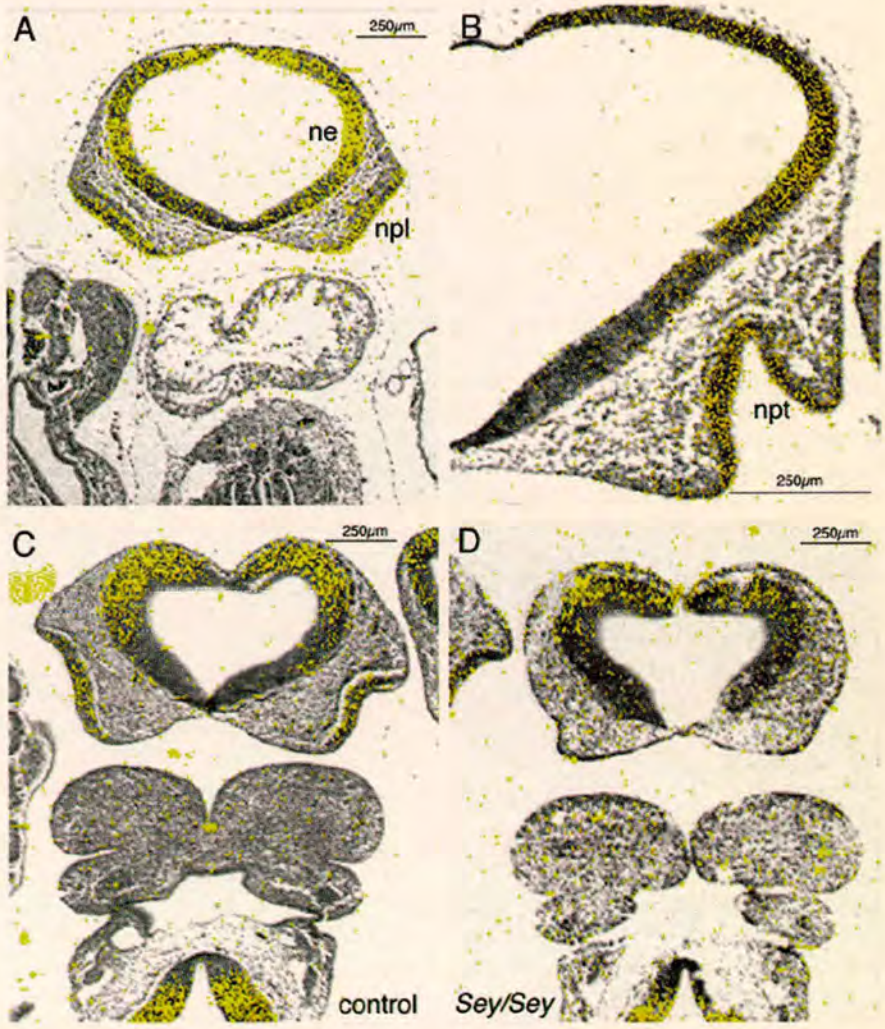

Fig. 6. Frontal sections through the nasal region showing *Pax-6* mRNA expression, yellow, in normal embryos at (A) E9.5 and (B) E10.5. Absence of nasal placode and nasal region expression at E9.75 from (D) *Sey/Sey* embryo, compare with (C) control embryo. Nasal placode (npl), nasal pit (npt), neuroepithelium of prosencephalon (ne).

from a lack of functional Pax-6 in the distal optic vesicle, where *Pax-6* mRNA is most strongly expressed.

Compared with littermates, optic vesicles of *Sey/Sey* embryos from E9.5 to E11.5 have a more uniform lumen width along their proximal-distal axis. *Sey/Sey* optic vesicles (E11.5 to E15.5) do eventually form structures reminiscent of both optic cup and optic stalk. These *Sey/Sey* structures vesicles retain some features of normal patterning, despite morphological abnormalities. *Tyrp2*, which in normal eyes is expressed only in the most distal optic stalk and the RPE layer of the optic cup, retains this distally restricted expression in *Sey/Sey* optic vesicles. As found in normal eyes, *Pax-6* transcripts in *Sey/Sey* optic vesicles are also most abundant in distal structures and are present in more extensive domains than those of *Tyrp2*.

Tyrp2 and *Pax-6* expression also highlight some abnormal features of *Sey/Sey* optic vesicles. Schmahl et al. (1993) suggested that the optic vesicle abnormalities could result from a disruption of proliferation or differentiation choices in the distal optic vesicle. Our findings support this suggestion. The small size of the expression domains of *Tyrp2* and *Pax-6* in *Sey/Sey* embryos may indicate that growth of the distal optic vesicle fails to keep pace with the development of other structures. This may explain the increasing separation of optic vesicle from the surface ectoderm. The two layers of the cup-like structure in *Sey/Sey* optic vesicles are morphologically similar and have similar expression characteristics, suggesting that differentiation of the developing cup into distinct layers is disrupted. Since there is no clear equivalent of the RPE, differentiation towards this cell type might be particularly affected in *Sey/Sey* mice.

Whilst the absence of lens in *Sey/Sey* mice complicates the identification of roles for *Pax-6* within the optic vesicle, it also allows us to use *Sey/Sey* mice to study the influence of the lens on optic vesicle development. Thus, those aspects of proximodistal patterning of the optic vesicle that are retained by *Sey/Sey* embryos do not depend upon the presence of a lens adjacent to the distal end of the optic vesicle.

Timing of Pax-6 action in lens formation

Lens 'induction' is a multi-step process (Grainger et al., 1988). The initiation of the lens formation pathway involves a series of inductive interactions prior to the contact between the surface ectoderm and the optic vesicle. Surface ectoderm passes through a progression of states of competence to respond to the different lens-inducing signals (Karlinen-Jääskeläinen, 1978; Henry and Grainger, 1987, 1990; Serventnick and Grainger, 1991; reviewed by Jacobson and Sater, 1988; Grainger, 1992). Although contact with the optic vesicle was long thought to be both necessary and sufficient to induce surface ectoderm to form lens (reviewed by Saha et al., 1989), it has been shown that the optic vesicle is not essential for initial formation of lens but can nevertheless induce lenses from ectoderm with lens-forming potential (Henry and Grainger, 1990) and is important for maintenance and growth of the lens (Saha et al., 1989).

Within the process of lens development, Pax-6 must act prior to, or at, the time of placode formation, i.e. before E9.5 in the mouse. Studies in amphibians suggest that the very earliest stages of lens induction occur around the time of gastrulation (Jacobson, 1966; Henry and Grainger, 1987). If comparable processes occur in mammalian eye development they are unlikely to involve Pax-6, since *Pax-6* is only expressed from the time (E8.0 in mouse) when the optic pits first appear. (Walther and Gruss, 1991; Krauss et al., 1991a,b; Puschel et al., 1992). Since surface ectoderm in *Sey/Sey* embryos fails to attain the earliest recognisable stage of lens formation, use of these animals does not address possible roles for *Pax-6* later in lens development. Nevertheless, Pax-6 is necessary for normal lens formation during the time, E8.0-E9.5, between the

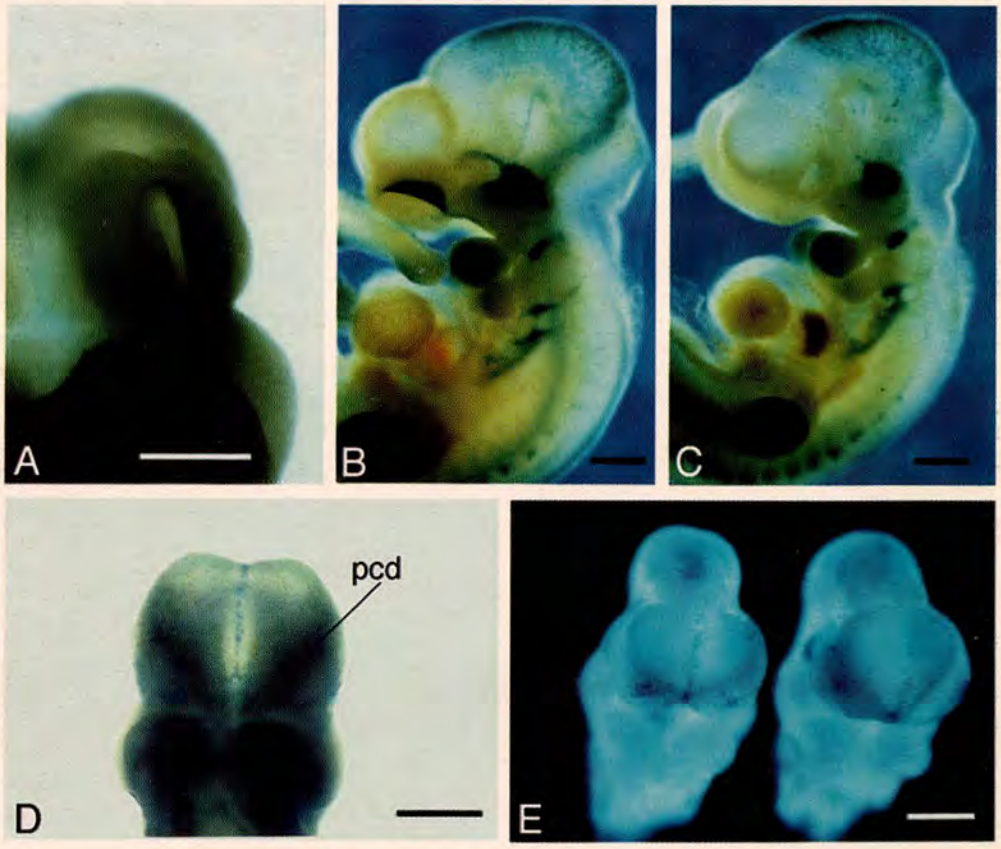

Fig. 7. (A-C) Expression of the *Msx-1* transgene ΔH6 at E10.5. Normal lateral placodal epithelium expression (A); control embryo (B); *Sey/Sey* embryo (C). (D-E) Nile Blue Sulphate (NBS) staining for programmed cell death. Arc of NBS-staining, programmed cell death (pcd), around rim of normal placode in D. (E) NBS staining in *Sey/Sey* embryo (left) and control embryo (right). All scale bars represent 1 mm.

first appearance of *Pax-6* expression and lens placode formation.

Pax-6 and lens determination

In normal development of the mouse eye, broad domains of *Pax-6* mRNA expression in head surface ectoderm are down-regulated, with expression being maintained in the developing lens placode. In the chick, the ability of isolated ectoderm to differentiate into lens in culture is initially a property of head ectoderm over a broad domain which, with time, also becomes restricted to the lateral regions adjacent to the optic vesicles (Barabanov and Fedtsova, 1982). Therefore, the surface ectoderm expression of the *Pax-6* gene and the *Sey/Sey* mutant phenotype are consistent with a role for Pax-6 in lens determination.

Tissue recombination experiments using the rat *Small eye* (Fujiwara et al., 1994) found that lens formation depended upon the genotype of the surface ectoderm used and was independent of the genotype of the optic vesicle. Thus *rSey/rSey* surface ectoderm never formed lens, whereas +/+ and *rSey*/+ surface ectoderm could form lens, even when cultured with *rSey/rSey* optic vesicle. It is possible that these results reflect a failure of signalling in vivo prior to the 20-somite stage when the recombinations were performed, but they are equally consistent with a requirement for functional Pax-6 within the surface ectoderm in order to form a lens placode and lens. Moreover, they further limit the role of the optic vesicle in the development of the homozygous mutant phenotype, as *rSey/rSey* optic vesicles can support the later development of the lens.

Pax-6 expression and ability to transdifferentiate to lens

Some non-lens tissues that express *Pax-6* are able to transdifferentiate into lens. The prospective pineal gland expresses *Pax-6* (Walther and Gruss, 1991), is multipotent and has the capacity to form lens (Watanabe et al., 1992). Similarly embryonic retina, iris and pigmented retinal epithelium at the anterior of the eye all express *Pax-6* mRNA and all have the ability to transdifferentiate into lens (reviewed by Okada, 1991). *Pax-6* expression may be a prerequisite for lens formation and, in non-lens tissue, may reflect a lens-forming ability that is normally overridden by diversion to other pathways.

We cannot rule out the possibility that the failure of lens formation in *Sey/Sey* embryos results entirely from a disruption of an interaction with the optic vesicle. An alternative explanation, Pax-6 involvement in lens determination, is supported by the association of *Pax-6* expression with ability of surface ectoderm to differentiate into lens, by the rat *Small eye* tissue recombination results and by the relationship between *Pax-6* expression and ability to transdifferentiate into lens. In addition, an activity of Pax-6 independent of the influence of the optic vesicle is revealed by down-regulation of *Pax-6* mRNA in *Sey/Sey* surface ectoderm.

Down-regulation of *Pax-6* mRNA in *Sey/Sey* surface ectoderm

Activation of *Pax-6* mRNA in *Sey/Sey* embryos is normal, but at the time when most surface ectoderm normally ceases to express *Pax-6*, the turn-off mechanism appears to extend to the whole surface ectoderm.

This extended down-regulation could be explained in a number of ways, but the observation that *Pax-6* mRNA continues to be expressed in normal chick surface ectoderm in the absence of an optic vesicle (Li et al., 1994) argues against failed signalling from optic vesicle and against a negative influence on *Pax-6* expression from mesenchymal-like cells gaining access to placode region surface ectoderm. We suggest a role for Pax-6 as an effector of its own expression in placode-forming ectoderm. Plaza et al. (1993) found a Pax-6-binding site in the quail *Pax-6* promoter through which Pax-6 up-regulated its own transcription in transfection assays, so the Pax-6-dependent regulation that we observe may reflect direct autoregulation.

Nasal placode formation and nasal processes

Both nasal cavities and lens develop from ectodermal placodes. We have found extensive similarities between the nasal lens placodes in their *Pax-6* expression and *Sey/Sey* mutant phenotype. Like the lens placode, the nasal placode normally expresses *Pax-6* mRNA and fails to form in *Sey/Sey* mutants. Similarly *Pax-6* mRNA expression turns off throughout the nasal region in *Sey/Sey* embryos. These results are consistent with the suggestion of Hogan et al. (1986) that the eye and nasal phenotype of *Sey/Sey* mice might stem from a common defect in the formation or early differentiation of these placodes.

The absence of nasal region *Msx-1* transgene expression in *Sey/Sey* embryos allows us to speculate that *Msx-1*, or genes regulating it, could be targets for Pax-6 in the nasal region. More concretely, absence of this expression shows that ectoderm in the nasal region of *Sey/Sey* embryos not only fails to attain the morphology characteristic of a placode, but also lacks gene expression normally associated with the nasal placode. Characteristic domains of morphological cell death, detected by Nile Blue Sulphate staining, are present in the nasal region of *Sey/Sey* embryos and control animals but, in *Sey/Sey* embryos, the domains are disorganised. Presence of these domains indicates that nasal region ectoderm is distinct from other head ectoderm, and so suggests that localisation of the nasal territory need not involve Pax-6 expression. Instead, Pax-6 may play a role in the transition from ectoderm to placode. From the similarity between lens and nasal placodes in terms of their normal development, *Sey/Sey* phenotype and *Pax-6* expression, we expect the requirement for Pax-6 in lens placode development to also be in the transition from ectoderm to placode.

We would particularly like to thank Richard Baldock for facilitating the computer presentation of expression data, Andrew Ross for help with the electron microscopy, Norman Davidson, Sandy Bruce and Douglas Stuart for photographic work, and the staff at the Biomedical Research Facility, Western General Hospital, Edinburgh for expert technical assistance. Ian Jackson and Albert E.Chung generously provided probes, and we thank Veronica van Heyningen for guidance and support of this work and Isobel Hanson for critical reading of the manuscript.

REFERENCES

Baldwin, C. T., Hoth, C. F., Amos, J. A., da Silva, E. O. and Milunsky, A. (1992). An exonic mutation in the *HuP2* paired domain gene causes Waardenburg's syndrome. *Nature* **355**, 637-638.

Barabanov, V. M. and Fedtsova, N. G. (1982). The distribution of lens differentiation capacity in the head ectoderm of chick embryos. *Differentiation* **21**, 183-190.

Beddington, R. S. P. and Lawson, K. A. (1990). Clonal analysis of cell lineages. In *Postimplantation Mammalian Embryos: a Practical Approach* (eds. Copp, A. J. and Cockroft, D. L.), pp. 267-292. Oxford University Press.

Bopp, D., Burri, M., Baumgartner, S., Frigerio, G. and Noll, M. (1986). Conservation of a large protein domain in the segmentaion gene *paired* and in functionally related genes of *Drosophila*. *Cell* **47**, 1033-1040

Chalepakis, G., Fritsch, R., Fickenscher, H., Deutsch, U., Goulding, M. and Gruss P. (1991). The molecular bais of the *undulated /Pax-1* mutation. *Cell* **66**, 873-884.

Coulombre, A. J. and Coulombre, J. L. (1964). Lens development: I. Role of the lens in eye growth. *J. Exp. Zool.* **156**, 39-48

Dong, L.-J. and Chung, A. E. (1991). The expression of the genes for entactin, laminin A, laminin B1 and laminin B2 in murine lens morphogenesis and eye development. *Differentiation* **48**, 157-172.

Epstein, D. J., Vekemans, M. and Gros, P. (1991). *Splotch* (Sp^{2H}), a mutation affecting development of the mouse neural tube, shows a deletion within the paired homeodomain of *Pax-3*. *Cell* **67**, 767-774.

Figdor, M. C. and Stern, C. D. (1993). Segmental organisation of embryonic diencephalon. *Nature* **363**, 630-634.

Frigerio, G., Burri, M., Bopp, D., Baumgartner, S. and Noll, M. (1986). Structure of the segmentation gene *paired* and the Drosophila PRD gene set as part of a gene network. *Cell* **47**, 735-746.

Fujiwara, M., Uchida, T., Osumi-Yamashita, N. and Eto, K. (1994). Uchida rat (*rSey*): a new mutant rat with craniofacial abnormalities resembling those of the mouse *Sey* mutant. *Differentiation* **57**, 31-38.

Glaser, T., Walton, D. S. and Maas, R. L. (1992). Genomic structure, evolutionary conservation and aniridia mutations in the human *PAX6* gene. *Nature Genet.* **2**, 915-920.

Graham, A., Heyman, I. and Lumsden, A. (1993). Even-numbered rhombomeres control the apoptotic elimination of neural crest cells from odd-numbered rhombomeres in the chick hindbrain. *Development* **119**, 233-245.

Grainger, R. M., Henry, J. J. and Henderson, R. A. (1988). Reinvestigation of the role of the optic vesicle in embryonic lens induction. *Development* **102**, 517-526.

Grainger, R. M. (1992). Embryonic lens induction: shedding light on vertebrate tissue determination. *Trends Genet.* **8**, 349-355.

Hanson, I. M, Seawright, A., Hardman, K., Hodgson, S., Zaletayev, D., Fekete, G. and van Heyningen, V. (1993). *PAX6* mutations in aniridia. *Hum. Molec. Genet.* **2**, 915-920.

Hanson, I. M., Fletcher, J. M., Jordan, T., Brown, A., Taylor, D., Adams, R. J., Punnett, H. and van Heyningen, V. (1994). Mutations at the *PAX6* locus are found in heterogeneous anterior segment malformations including Peters' anomaly. *Nature Genet.* **6**, 168-173.

Heinzmann, U., Favor, J., Plendl, J. and Grevers, G. (1991). Enticklungsstorung des olfaktorischen Organs. Ein Beitrag zur kausalen Genese beieiner Mausmutante. *Verh. Anat. Ges.* **85**, (Anat. Anz. Suppl. 170), 511-512.

Henry, J. J. and Grainger, R. M. (1987) Inductive interactions in the spatial and temporal restriction of lens-forming potential in embryonic ectoderm of *Xenopus laevis*. *Dev. Biol.* **24**, 200-214

Henry, J. J. and Grainger, R. M. (1990) Early tissue interavtions leading to embryonic lens formation in *Xenopus laevis*. *Dev. Biol* **141**, 149-163

Hill, R. E., Favor, J., Hogan, B. L. M., Ton, C. C. T., Sauders, G. F., Hanson, I. M., Prosser, J., Jordan, T., Hastie, N. D. and van Heyningen V. (1991). Mouse *Small eye* results from mutaions in a *paired*-like homeobox-containing gene. *Nature* **354**, 522-525.

Hogan, B. L. M., Horsburgh, G., Cohen, J., Hetherington,C. M., Fisher, G. and Lyon, M. F. (1986). *Small eyes* (*Sey*): a homozygous lethal mutation on chromosome 2 which affects the differentiation of both lens and nasal placodes in the mouse. *J. Embryol. Exp. Morph.* **97**, 95-110.

Jacobson, A. G. (1966). Inductive processes in embryonic development. *Science* **152**, 25-52.

Jacobson, A. G. and Slater, A. K. (1988). Features of embryonic induction. *Development* **104**, 341-359.

Jordan, T., Hanson, I., Zaletayev, D., Hodgson, S., Prosser, J., Seawright, A., Hastie, N. and van Heyningen, V. (1992). The human *PAX6* gene is mutated in two patients with aniridia. *Nature Genet.* **1**, 328-332.

Karkinen-Jääaskeläinen, M. (1978). Permissive and directive interactions in lens induction. *J. Embryol. Exp. Morph.* **44**, 167-179.

Krauss, S., Johansen, T., Korzh, V. and Fjose, A. (1991a). Expression pattern of zebrafish *pax* genes suggests a role in early brain regionalization. *Nature* **353**, 267-270.

Krauss, S., Johansen, T., Korzh, V., Moens, U., Ericson, J. U. and Fjose, A. (1991b). Zebrafish *pax*[*zf-a*]: a paired box-containing gene expressed in the neural tube. *EMBO J.* **10**, 3609-3619.

Li, H.-S., Yang, J.-M., Jacobson, R. D., Pasko, D. and Sundin, O. (1994). *Pax-6* is first expressed in a region of ectoderm anterior to the early neural plate: implications for stepwise determination of the lens. *Dev. Biol.* **162**, 181-194.

Lyon, M. F. and Searle, A. G. (1989). *Genetic Variants and Strains of the Laboratory Mouse*. (2nd ed.). Oxford Univeristy Press.

Matsuo, T., Osumi-Yamashita, N., Noji, S., Ohuchi, H., Koyama, E., Myokai, F., Matsuo N., Taniguchi S., Doi H., Iseki S., Nimomiya Y., Fujiwara M., Watanabe T. and Eto K. (1993). A mutation in the *Pax-6* gene in rat *small eye* is associated with impared migration of midbrain neual crest. *Nature Genet.* **3**, 299-304.

Monaghan, A. P., Davidson, D. R., Sime, C., Graham, E., Baldock, R., Bhattacharya, S. S. and Hill, R. E. (1991). The *Msh*-like homeobox genes define domains in the developing vertebrate eye. *Development* **112**, 1053-1061.

Okada, T. S. (1991). *Transdifferentiation: Flexibility in Cell Differentiation.* Oxford University Press, Oxford.

Plaza, S., Dozier, C. and Saule, S. (1993). Quail *PAX-6* (*PAX-QNR*) encodes a transcription facor able to bind and trans-activate its own promoter. *Cell Growth Differ.* **4**,1041-1050.

Puelles, L. and Rubenstein, J. L. R. (1993). Expression of homeobox and other putative regulatory genes in the embryonic mouse forebrain suggests a neuromeric organization. *Trends Neurosci.* **16**, 472-479.

Puschel, A. W., Gruss, P. and Westerfield, M. (1992). Sequence and expression pattern of *pax-6* are highly conserved between zebrafish and mice. *Development* **114**, 643-651.

Saha, M. S., Spann, C. L. and Grainger, R. M. (1989). Embryonic lens induction: more than meets the optic vesicle. *Cell Different. Dev.* **28**, 153-172.

Schmahl, W., Knoedlseder, M., Favor, J. and Davidson, D. (1993). Defects in neuronal migration and the pathogenesis of cortical malformations are associated with *Small eye (Sey)* in the mouse, a point mutation at the *Pax-6* locus. *Acta Neuropathol.* **86**, 126-135.

Servetnick, M. and Grainger, R. M. (1991) Changes in neural and lens competence in Xenopus ectoderm: evidence for an autonomous developmental timer. *Development* **112**, 177-188

Steel, K. P., Davidson, D. R. and Jackson, I. J. (1992). TRP-2/DT, a new early melanoblast marker, shows that the steel growth factor (c-kit ligand) is a survival factor. *Development* **115**, 1111-1119.

Sulik, K. K., Cook, C. S. and Webster, W. S. (1988). Teratogens and craniofacial malformations: relationships to cell death. *Development* **103 Suppl.**, 213-232.

Tassabehji, M., Read, A. P., Newton, V. E., Harris, R., Balling, R., Gruss, P. and Strachan, T. (1992). Waardenburg's syndrome patients have mutations in the human homologue of the *Pax-3* paired box gene. *Nature* **355**, 635-636.

Ton, C. C. T., Hirovenen, H., Miwa, H., Weil, M. W., Monaghan, A. P., Jordan, T., van Heyningen, V., Hastie, N. D., Meijers-Heijboer, H., Drechsler. M,. Royer-Pokora, B., Collins, F., Swaroop, A., Strong, L. C. and Saunders, G. F. (1991). Positional cloning and characterization of a paired box and homeobox containing gene from the aniridia region. *Cell* **67**, 1059-1074.

Treisman, J., Harris, E. and Desplan, C. (1991). The paired box encodes a second DNA-binding domain in the Paired homeodomain protein. *Genes Dev.* **5**, 594-604.

Walther, C. and Gruss, P. (1991). *Pax-6*, a murine paired box gene, is expressed in the developing CNS. *Development* **113**, 1435-1449.

Walther, C., Guenet, J. L., Simon, D., Deutsch, U., Jostes, B., Goulding, M. D., Plachov, D., Balling, R. and Gruss, P. (1991). Pax: a murine mutigene family of paired box-containing genes. *Genomics* **11**, 424-434.

Watanabe, K., Araki, M. and Iwasaki, H. (1992). The embryonic pineal body as a multipotent organ. *Microscopy research and technique* **21**, 218-226.

(Accepted 31 January 1995)